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Defining Inherited Haematological Disease within the Irish Traveller Population.

Thesis submitted for the degree of Doctor of Medicine

University of Dublin

Trinity College

2005

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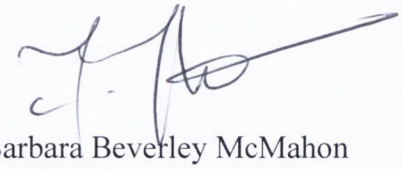

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SUMMARY

The Traveller Community in Ireland constitutes a minority of nomadic or semi-nomadic people, sharing a common Irish ancestry. The Traveller Health Study showed that in 1987, Travellers were only reaching the life expectancy that the settled population had reached in the 1940's and that the perinatal mortality for travellers was as high as the national rate in 1966

Aims of the Present study.

Little is known within the general medical community about the specific genetic diseases affecting Traveller children. The aim of this study was to identify, in particular, those diseases presenting to the Haematologist and to define their natural history. The study evaluated current treatment practice and proposed changes based on the study's findings. It examined the availability of Haematopoietic Stem Cell Transplantation and investigated strategies which might make it easier to identify a donor. It is hoped that this study will raise awareness of these conditions in the Traveller population facilitating earlier diagnosis and treatment.

Methodology

An analysis of the currently available data on Traveller Health was undertaken. A review of the Health care strategies instituted within the last 15 years was undertaken. A questionnaire was then sent to all Paediatricians and Haematologists in the Republic of Ireland to identify the types of diseases found in the Traveller community. An analysis of the databases from the 2 Tertiary Haematology Centres was undertaken and with the help of the National Centre of Medical Genetics a table of the genetic diseases found in this population was established.

Diseases with specific haematological associations were identified and analysed in detail. Charts were reviewed, treatment strategies identified and outcomes recorded. Genotype-phenotype relationships were identified, if possible, as many of the diseases had novel mutations. Review of international literature was undertaken and "best practice" strategies were constructed. Laboratory methods were applied to rare diseases to facilitate disease recognition and earlier treatment.

The work-books of the HLA laboratory, Irish Blood Transfusion Service were analysed and raw HLA data extracted for those Traveller children who had bone marrow donor searches undertaken, and family members who also had HLA typing performed. A database was constructed for the Traveller group. The availability of donors from within the traveller group, the Irish unrelated bone marrow donor and world-wide donor panel was assessed and any unique HLA phenotypes existing within the Traveller group identified. Haplotypes were derived from all of the traveller data and this was compared with known haplotypes from the general Irish population again, identifying unique characteristics.

Major findings

This is the first time that a comprehensive list of diseases affecting specific Traveller families has been compiled. Six diseases were identified with specific haematological associations; Cytochrome B5 Reductase deficiency, Fanconi Anaemia (FA), Severe Factor X deficiency, Haemophagocytic Lymphohistiocytosis (HLH), Hurler syndrome and Carbonic Anhydrase II deficiency (CAII). The 5 most common were evaluated fully in this study, whilst Cytochrome B5 reductase deficiency has been the subject of a collaborative paper.

New gene mutations have been identified in Traveller associated Fanconi Anaemia (FANCA *del11-14*), Severe Factor X deficiency (Pro382Leu) and Carbonic Anhydrase II deficiency (*Del12/ins4 Exon6).*

The natural history of severe factor X deficiency due to Pro382Leu mutation was identified. This produces a severe bleeding disorder which may be fatal. Families at risk can be identified, cord blood sampling at birth, for factor X levels, undertaken and prophylaxis instituted in the neonatal period. Central venous Access devices can be sited following correction of the haemostatic deficit using prothrombin complex concentrate. The half-life of Factor X is variable and fall-off studies must be undertaken to ensure adequate prophylaxis is obtained.

The natural history of FA in the Travellers has been defined. All have short stature and many had been investigated for "failure to thrive" prior to diagnosis. Bone marrow failure is inevitable and occurs between 40-65 months of age. This is an unusual finding in most individuals with FANCA genotype. Bone marrow failure occurring prior to 40 months may be associated with rapid development of acute leukaemia.

Haemophagocytic lymphohistiocytosis can occur in specific Traveller populations and appears related to Natural Killer Cell deficiency. There may be a link with Fanconi Anaemia. Perforin analysis and NK cell measurement have been identified as useful laboratory diagnostic aids. Early Bone Marrow transplantation may be curative.

Hurler syndrome caused by W402X gene mutation is common in some Traveller families. Contrary to published data, transplantation should be undertaken before the age of 12 months for maximum effect. Data from this study has lead to a change in Transplantation policy and unrelated donors, if readily available are now used in preference to prolonged extended family donor searching.

Analysis of Traveller HLA phenotypes and haplotypes identified unique characteristics. The institution of a specific Traveller cord blood bank would provide a greater possibility of finding a haematopoietic donor but the development of isolated community specific cord banks could not be recommended. Directed cord donation for families "at risk" could be considered.

Abbreviations used in this Text

AAP	age at presentation
A,	associated with amyloidosis
A*,	secondary amyloidosis
ACD	Acid Citrate Dextrose
AN	abnormal
Ab†,	extremely low specific activities
ADCC	antibody-dependant cellular cytotoxicity
Ag	antigen
ALT	alanine transferase
AML,	acute myeloid leukaemia
AMNCH,	National Children's Hospital Tallaght
APTT,	activated partial thromboplastin time
ASD/LVH,	atrial septal defect & left ventricular hypertrophy
AT	Ataxia- telangiectasia
ATG	anti-thymocyte globulin
α_1AT,	Alpha 1 anti-trypsin deficiency
B,	Busulphan
BMA,	bone marrow aspirate
BMDW,	Bone Marrow Donors Worldwide
BMT,	bone marrow transplantation
BSER	brainstem evoked responses
Ca++	calcification
CA,	Carbonic Anhydrase
CAI,	carbonic anhydrase I
CAII	carbonic anhydrase II
CB5R,	Cytochrome B5 Reductase
CF,	cystic fibrosis
CSF,	cerebrospinal fluid
CTLs,	cytotoxic T lymphocytes
CVA,	cerebrovascular accident
CVAD,	Central Venous Access Devices
Cy,	cyclophosphamide
D,	different donor
DEB,	Diepoxybutane
DK,	do not know
EDTA,	Ethylene diamine Tetraacetic acid
ERG,	electroretinogram
F,	female
(F)	factor X Friuli variant
FA,	Fanconi Anaemia
FBC,	full blood count estimation
FD,	family donor
FGF,	ibroblast growth factor
FH,	family history
FFP,	fresh frozen plasma
FLH,	familial HLH
Fr,	frequency

FTT,	failure to thrive
FVC,	Forced Vital capacity
FX,	factor ten
FXAg,	Factor X antigen
FXPT,	factor X measured by PT methodology
FXPT:APTT,	comparison of PT based assay with values obtained using APTT methodology
FXRVV,	Factor X measured using Russell Viper venom
g/dl,	grams per decilitre
g/l,	grams per litre
GAG,	glycosaminoglycans
GM,	Grandmother
GM-CSF,	granulocyte-macrophage colony-stimulating factor
GMDS	Griffiths mental development scale
GMS,	General Medical Scheme
GPs,	General Practitioners
GVHD,	graft versus host disease
H,	hereditary
HB,	Health Board
Hb,	haemoglobin
Het.,	heterozygous
HF,	Haplotype Frequency
HGT	height
HIT,	Haploidentical transplant
HLA,	Human Leucocyte antigen (HLA)
HLH,	Haemophagocytic Lymphohistiocytosis
HSCT,	Haematopoietic Stem Cell Transplantation
I,	improved
IBTS,	Irish Blood Transfusion Service
IFN,	interferon
IFN-γ,	interferon γ
IL,	interleukin
IrBMR,	Irish Bone Marrow Donor Registry
IVIg,	intravenous immunoglobulin
IQ	intelligence quotient
L	left
LDH,	lactate dehydrogenase
lu,	lumbar
LVH,	left ventricular hypertrophy
M,	male
M-CSF,	macrophage colony stimulating factor
MDS,	myelodysplastic syndrome
Meth-Hb,	Methaemoglobinaemia
Mg/kg,	milligrams per kilogram
MHC,	Major Histocompatiblility Complex
MMC,	Mitomycin C
Mo,	months
MPS,	mucopolysaccaridoses
MR,	mitral regurgitation
MR/AR,	mitral regurgitation & aortic regurgitation

MS	millisecond
MTX,	methotrexate
MUD,	matched unrelated transplant
MVT,	mitral valve thickening
MW,	minimal worsening
N,	normal/no
N/A,	not available
NBS,	Nijmegen breakage syndrome
NC,	unchanged
Neurod.,	neurodevelopment
NK,	Natural killer cells
No.,	number
NR,	not relevant
ODF,	osteoclast differentiation factor
OLHSC,	Our Lady's Hospital for Sick Children Crumlin
OP-	osteopetrosis
OPG,	osteoprotegerin
PBSCT,	Peripheral Blood Stem Cell Transplant.
PCC,	prothrombin complex concentrates (PCC).
PDA,	Patent Ductus Arteriosus
PDGF,	platelet derived growth factor
PTHrP,	PTH-related protein.
R,	resolved
RCC	red cell concentrate
Rt	right
RTA	renal tubular acidosis
RVVT,	Russell viper venom time
S,	successful
SCID	Severe Combined Immunodeficiency
SD	Same Donor
SEHB,	South Eastern Health Board
SHB,	Southern Health Board
Sib,	sibling
SS,	short stature
ST,	Sibling transplant
T,	Traveller
T/A,	transiently acquired
TAI,	thoraco abdominal irradiation (TAI)
TAR,	Thrombocytopenia–absent radii
TGFβ,	transforming growth factor-β
Th,	thoracic
Th/lu,	thoracolumbar
TNF,	tumour necrosis factor
TNF-α,	tumour necrosis factor α
Tr,	transplant
TR,	Traveller Registry
TSH,	Temple Street Children's Hospital;
$T_{1/2,}$	plasma half-life
U	Unit
UCT,	unrelated cord transplantation

u/l,	units per litre
VER	visual evoked responses
VOD,	veno-occlusive disease
W,	worsened
W+,	much worse
WBC,	white blood cell count
WHB,	Western Health Board
WHO,	World Health Organisation
Wk,	weeks
Wt	weight
Y,	yes
Yr	Year
>,	greater than
<,	less than
≤	less than or equal to.
2°,	secondary
µmol/l,	micromoles per litre

Publications arising from this study

Bone Marrow transplantation corrects osteopetrosis in the carbonic anhydrase II deficiency syndrome.
C.McMahon, A. Will, Peiyi Hu, GN Shah, WS Sly, OP Smith. Blood 2001;97:7:1947-1950

The Role of Primary Prophylactic Factor Replacement Therapy in Children with Severe Factor X Deficiency.
Mc Mahon C, Smith J, Goonan C, Byrne M, Smith OP. British Journal of Haematology 2002; 199; 789 – 791

Recessive congenital methaemoglobinaemia: functional characterisation of the novel D239G mutation in the NADH-binding lobe of cytochrome b5 reductase.
MJ Percy, LJ Crowley, CA Davis, MF McMullin, G Savage, J Hughes, C McMahon, RJM Quinn, O Smith, MJ Barber TRJ Lappin. British Journal of Haematology. 2005;129:847-853

Publications in preparation

The natural history of Fanconi Anaemia in an Irish population
C McMahon, A O Marcaigh, OP Smith

Natural Killer Cell Deficiency and haemophagocytic Lymphohistiocytosis
C McMahon, A O Marcaigh, S Rooney, OP Smith

Laboratory measurement of Factor X
C McMahon, J Uprichard, M Byrne, D Perry, OP Smith

Haplotypes and phenotypes on an Irish population
C McMahon, J Crowley, C Dunne, OP Smith, E Lawlor

Chapter 1

History and demographics of the Irish Traveller

The Traveller population of contemporary Europe includes the Manouche in France, Belgium and Holland; the Sinti and Jenisch of Germany and German speaking countries; the Mercheros of Spain and the Reisende and Taterne of Norway. Estimates of Europe's Traveller population vary greatly from 2 million to 5 million. Not all of these are totally nomadic. It is estimated that only 25% are completely nomadic, 30% are semi-nomadic whilst almost 40% are sedentary or only irregularly nomadic. (McLoughlin, 1995). Liegeois in his report for the council of Europe states that "Nomadism is more a state of mind than an actual situation. Its existence and importance are frequently more psychological than geographical" (Liegeois, 1987)

The Irish Traveller population is a unique group within the Irish population with their own traditions and language. They are a nomadic people whose life style has changed in recent years. Many are now settled or semi-nomadic and very few are completely nomadic. In the 1960's Government policy was to assimilate Travellers into the settled community, a policy which was doomed to failure, whereas there is now official recognition of Travellers as a distinct minority with their own culture and beliefs. (Traveller Health, a national Strategy 2002-2005). Section 2(1) of the Equal Status Act 2000 contains an explicit definition of "Traveller community" which states: " 'Traveller community' means the community of people who are commonly called Travellers and who are identified (by both themselves and others) as people with a shared history, culture and traditions including, historically, a nomadic way of life on the island of Ireland".

Origins

"The truth is that we're a people like everybody else but we're a different-speaking people with our own traditions and our own way of life and this is the way we should be treated,.....not like dirt or drop-outs from the settled community. Some of my ancestors went on the road in the Famine but more of them have been travelling for

hundreds of years..... The Travellers have been in Ireland since St Patrick's time.....The original travellers were tinsmiths and musicians and they were great carpenters, they made all their own musical instruments and the wagons and carts." (Joyce N, 2000)

Nomadism and the Traveller communities have been deeply embedded in European society since the 5th Century AD. The most well known Traveller group are the Romanies or Gypsies. They migrated from India through Persia and into Europe between the 5th and the 13th centuries. Other groups of commercial nomads; Soothsayers, Magicians and Sorcerers came from India via Asia Minor early in the 9th Century. This was followed in the 10th Century by another major migration of nomadic people from India, many of whom settled in the Balkan states, whilst others migrated across Europe and arrived in England and Scotland in the early 16th century. (Kendrick D, 1996)

Astrology, witchcraft, magical healing and divination, always closely associated with the Travelling communities, were taken seriously in pre-Reformation Europe but the rise of science and growth of urban living was a serious set back for the travelling communities and undermined their strategic positions in pre-renaissance Europe. Periods of famine, economic depression and the Black Death lead to scapegoating of those who were considered "outsiders", including Jews, Muslims and the Travelling peoples. (McLoughlin J 1995). Laws were passed expelling Gypsies from many Western European countries and by the mid-sixteenth century some states had enacted laws making it a capital offence to be a Gypsy. (Acton T, 1996). The first anti-Gypsy law in England was passed in 1530 during the reign of Henry VIII. (Van Cleemput P,2000)

Irish Travellers are a distinct group from other gypsies and appear to be an endogenous rather than exogenous group. There are small groups of Travellers of Irish descent in Europe and United States but the majority are confined to Ireland and Britain. (McLoughlin J, 1995). The American Travellers are scattered across the southern states of Alabama, Georgia, Tennessee, South Carolina, Mississippi, Louisiana and Texas. They are the descendants of Irish Travellers who were part of the mass emigration from Ireland during the 1845-48 Famine. (Lockwood et al, 1994) The origins of the Irish

traveller are obscure because they have no written records and do not feature to any major extent in the major celtic warrior epics. It appears certain that not all families originated at the same time or in the same way. Some travelling families date back centuries whilst others adopted the nomadic life in recent times. Some probably began as itinerant craftsmen and specialists who travelled because of the limited demand for their work in any one place. Others were probably originally peasants and labourers who voluntarily went on the road to look for work or else were forced onto it by eviction or some other personal reason e.g. marriage to a "tinker". "Tinkers" and "tynkere" first appear as trade or surnames in written records in the 12th century but as early as the 5th century itinerant whitesmiths as well as other craftsmen and specialists such as tanners, musicians and bards travelled the Irish countryside fashioning jewellery, weapons and horse trappings out of bronze, silver and gold in exchange for food and lodgings. Tinkers were numerous enough in Ireland and Scotland to give Gypsies stiff competition when they arrived for the first time in the 16th century. (Gmelch, 1986; McLoughlin, 1995).

In the early years of the 20th century the Traveller was rural based and travelled from village to village making and repairing tin ware, cleaning chimneys, horse and donkey trading and working as a farm labourer at harvest time. They tended to camp on the road side in dispersed groups of 1-3 families from mid-March to November and then moved back to their home village or took refuge in abandoned houses in the country side. They seldom remained in one place longer than a few weeks and sometimes only for a day or two. The rapid industrialisation and mechanisation which occurred after the 2nd World War brought an end to many of their trades and most families moved to the cities and larger towns where they have remained in an uneasy truce with the settled community. (Gmelch S 1986)

Traditions and Culture.

Language

Travellers have their own language known as cant or gammon. It is also called Shelta in academic circles. It is not taught in schools but is known to all travellers. Children generally learn the language in infancy along with English as a first joint language. It

has a simplified English grammatical structure and much of the vocabulary consists of Irish words that have been transformed or disguised using a series of recent changes. It first came to light in the 1880's but is thought to be very old, possibly dating from before the 12th century and not related to Romany. (Binchy A, 1996)

The Family

Traveller groups tend to see themselves in terms of the extended family. Part of the reason for travelling is said to be to keep up with the news of the family and to meet up with other family members. This has a major social role as it allows parents to arrange the marriage of their children within the extended family. (Mc Donagh M, 1996) The Traveller community perceives substantial economic and social advantages to these consanguineous unions. The incidence of first cousin marriages in the Traveller population has been reported at 19% compared with 0.16% in the settled population (Barry et al, 1997). The issue of consanguinity is one that has received much attention within the community and is the subject of considerable debate.

Traveller membership is based on descent. A person has to have at least one traveller or gypsy parent to claim membership of the Travelling Community. The children of a "mixed" marriage between a traveller and a settled person can claim rights through descent. Even then the "settled" parent whilst gaining acceptance through marriage could not claim full traveller identity.

Nomadism

Nomadism in the context of the Irish traveller has been described as a way of looking at the world and incorporates a different attitude to accommodation, work and life. (Mc Donagh, 1996) Travellers view accommodation as a stopping place only and it is always a temporary measure whether the duration of the stay is for a long or short time. The realisation that the settled community expects the traveller to remain in the one place, possibly for the rest of their lives, is described as a terrifying one because it is said they always have a nomadic wish.

Nomadism serves many social functions. It helps people "keep up with the news", build contacts, strengthen relationships and arrange marriages. It also allows people to avoid one another if arguments arise.

Nomadism is part of the economic tradition. In the past it has allowed individuals to move to find work. Self employment is an essential part of traveller life. Travelling allows people to attend markets and fairs in order to "deal" and peddle their wares.

The ability of Travellers to travel has been threatened in recent years by a series of laws. The UK Government passed the Criminal Justice and Public Order Act in 1994 which removed the right to travel and introduced new penalties for travelling whilst here in Ireland new Trespass laws have made it more difficult to find halting places.

Traveller Economy. (Report of the Task Force on the Travelling Community, 1995)

Traveller economic life differs from that of the Settled Community in a number of respects:

- *Flexibility*. In contrast to the more career orientated approach of the "settled community" the Traveller will move from one economic activity to another as opportunities arise to make a profit.

- *Self-employment*. This is one of the most marked elements in the Gypsy or Traveller identity.

- *Income Base*. The Traveller economy is based on immediate payment for goods or services provided. This contrasts with an emphasis on jobs or job creation in the settled community.

The Irish Traveller Population

The true number of Travellers is unknown. It was estimated from Department of Environment and Local Government data in 2000 that there were 24,000 Travellers representing 4,898 Families living in Ireland. Central Statistics Office Census returns for 1996, which are now acknowledged to be inaccurate, suggested that the average Traveller family size was 5 people. (Central Statistics Office, 1996). The majority of families (1353) were located in the Eastern Regional Health Authority region. The Western, Mid-Western, Southern and South Eastern regions had a further 2419 families (Fig 1.1). Over 50% of all Travellers were found in one of 4 counties: Cork, Dublin, Galway and Limerick. (Barry et al, 1988)

The Traveller population has grown more than fourfold in the last 40 years. The median age of Travellers in 1996 was 14 years compared with 31 years for the national figure. (Fig 1.2) This may be due in part to a lower life expectancy in travellers and to a higher birth rate; 3.5 children compared with 1.8 in the General Population. (Traveller Health, a National Strategy 2002-2005 Dept of Health and Children). Data from a study commissioned by the Health Research Board in 1986-1987 on Traveller Health demonstrated that 3.4% of babies were born to single Traveller woman compared with 10.8% of babies born to single settled women. The average age of marriage for the Traveller women who gave birth in 1997 was 19 years. The fertility rates for "unhoused" teenage Travellers were 7 times higher than that of "housed" Traveller women. Amongst Travellers in their 20's the fertility rate was 2.5 times higher in unhoused versus housed traveller females and the trend continued for traveller females in there 30's were the fertility rate was still twice as high. (Barry et al, 1997)

Life Expectancy

The Traveller Health Study showed that in 1987 Travellers were only reaching the life expectancy that the settled population had reached in the 1940's. The life expectancy at birth for a Traveller male was 61.7 years and that of a Traveller female was 65.3 years compared with 71.6 and 77.2 years for males and females from the general population (Fig 1.3). Major causes of death in Travellers in 1987 were injury and poisoning (21%)

and diseases of the circulatory system (26%). Deaths from metabolic diseases and immune disorders accounted for 3.5% of all deaths and there were no reported deaths from diseases of blood or blood forming organs. (Barry et al, 1987)

Mortality rates in early life (Fig 1.4).

The stillbirth rate for Travellers in 1987 was 19.5 per 1000 total births compared to the national figure of 6.9/1000. The perinatal mortality rate was 28.3/1000 total births compared to a national figure of 9.9/1000. This figure was made up of 11 stillbirths and 5 early neonatal deaths in the Traveller group. The infant mortality rate for Travellers was 18.1/1000 live births compared to a national figure of 7.4. These data demonstrated that the perinatal mortality for travellers in 1987 was as high as the national rate in 1966. No comment could be made as to whether the type of accommodation had any part to play in these statistics. (Barry et al 1997).

Access to Health Care

A survey carried out by University College Galway in 1995 found that 83% of Travellers surveyed had medical cards but 17% found it difficult to get GPs to accept them onto their GMS lists. Thirty six percent experienced difficulties getting GPs to come on house calls. Twenty percent of the group had more than 10 consultations within the pervious year and 41% had received more than 10 prescriptions within the same time period. (Report of the Task Force on the Travelling Community, 1995). A recent study undertaken by the Traveller unit of the Eastern Region in association with Tallaght Hospital looked at the use of hospital facilities by the Traveller Community. Sixty three percent of children attending Tallaght hospital from June 1998 to May 1999 were from non-permanent sites which would have lacked water and sanitation. The Traveller children had twice as many admissions with gastroenteric problems as the children from the settled community and had almost three times as many respiratory problems. The main source of referral to outpatients was via Accident and Emergency Departments in the case of travellers whilst those of the settled community were more likely to be referred by their GP. Fifty percent of settled children had recently been seen

in outpatients but only 15% of Traveller children. (Report of Use of Hospital Facilities by the Travelling Community, 2000)

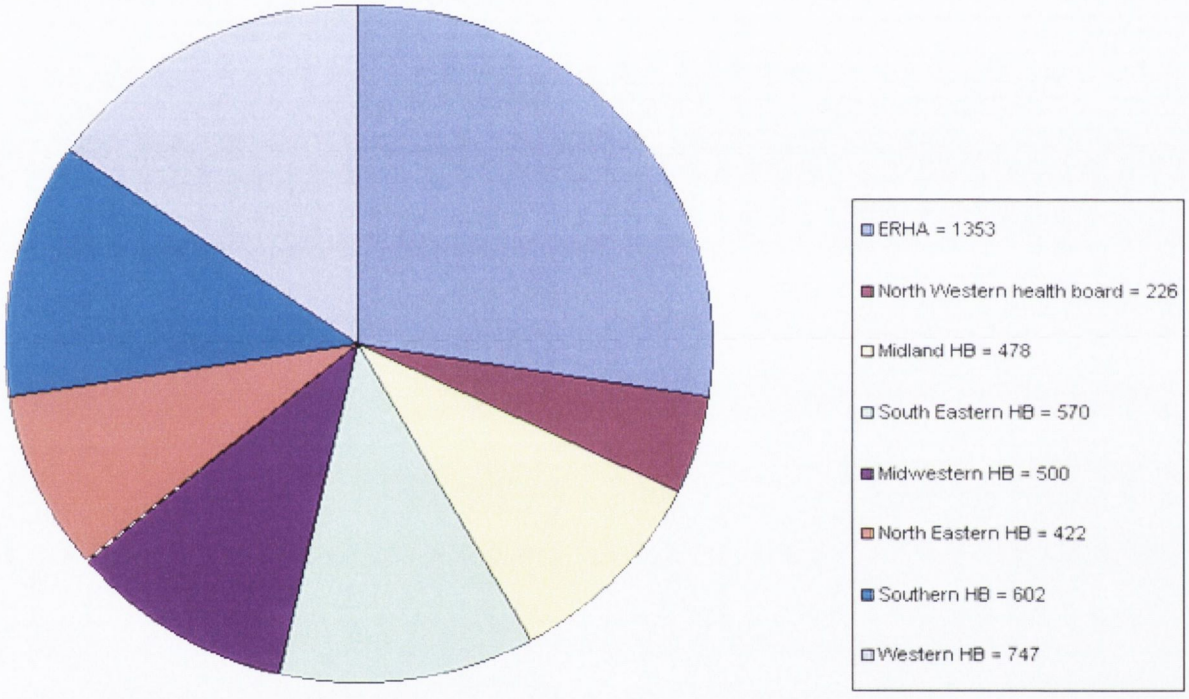
Aims of the Present study.

Little is known within the general Paediatric Medical Community about the specific diseases affecting members of the Traveller Community. There is even less knowledge about diseases affecting the bone marrow. Some diseases whilst not primarily haematological can benefit from bone marrow or haematopoietic stem cell transplant. The aim of this study is to identify those haematological diseases or associated conditions which affect the Irish Traveller population and to study their effect on the population. It will evaluate type and success of the available treatments. It will look at the availability of bone marrow transplant and investigate strategies which might make it easier to identify a bone marrow donor. It is hoped that this study will raise awareness of these conditions in the Traveller population facilitating earlier diagnosis and treatment in order to prevent some of long term adverse sequelae of these diseases.

Ethical approval.

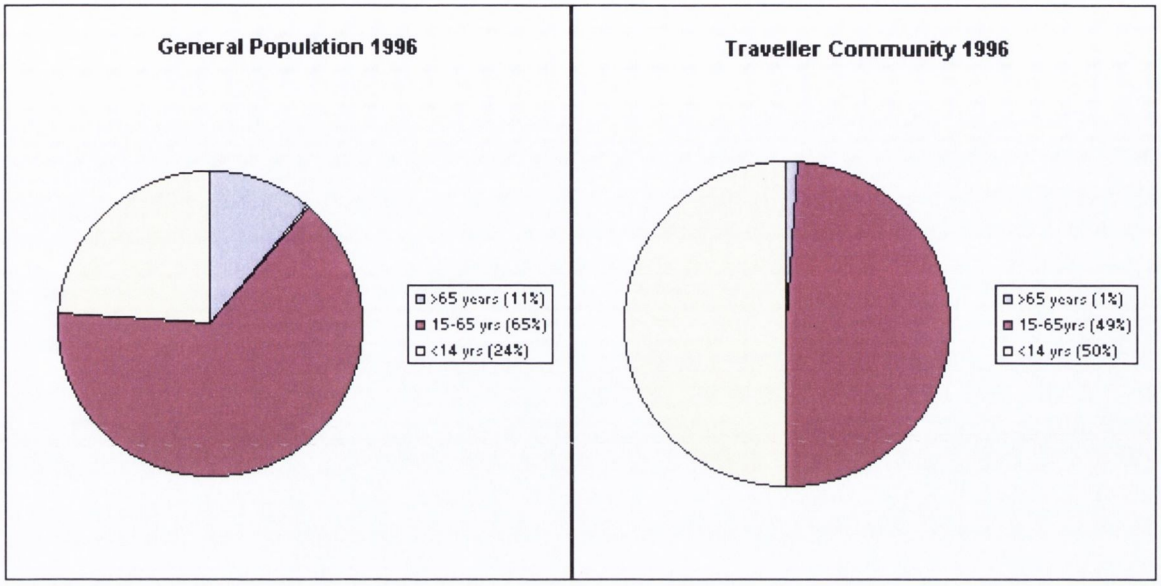
Ethical approval for this study was sought and considered by the St James's Hospital and Federated Dublin Voluntary Hospitals Joint Research Ethics Committee at its meeting on 28th August 2001. (Appendix 1 Application for Ethics approval). Approval for the study was granted if 5 specific points were addressed. (Appendix 2 letter of approval with conditions 3rd September 2001) These points were addressed in correspondence in January 2002 (Appendix 3), and full ethical approval was granted on 18th February 2002 (Appendix 4).

Fig 1.1 Number of Families by Health Board Region in 2000



From Traveller Health A national Strategy 2002-2005, Dept of Health and Children

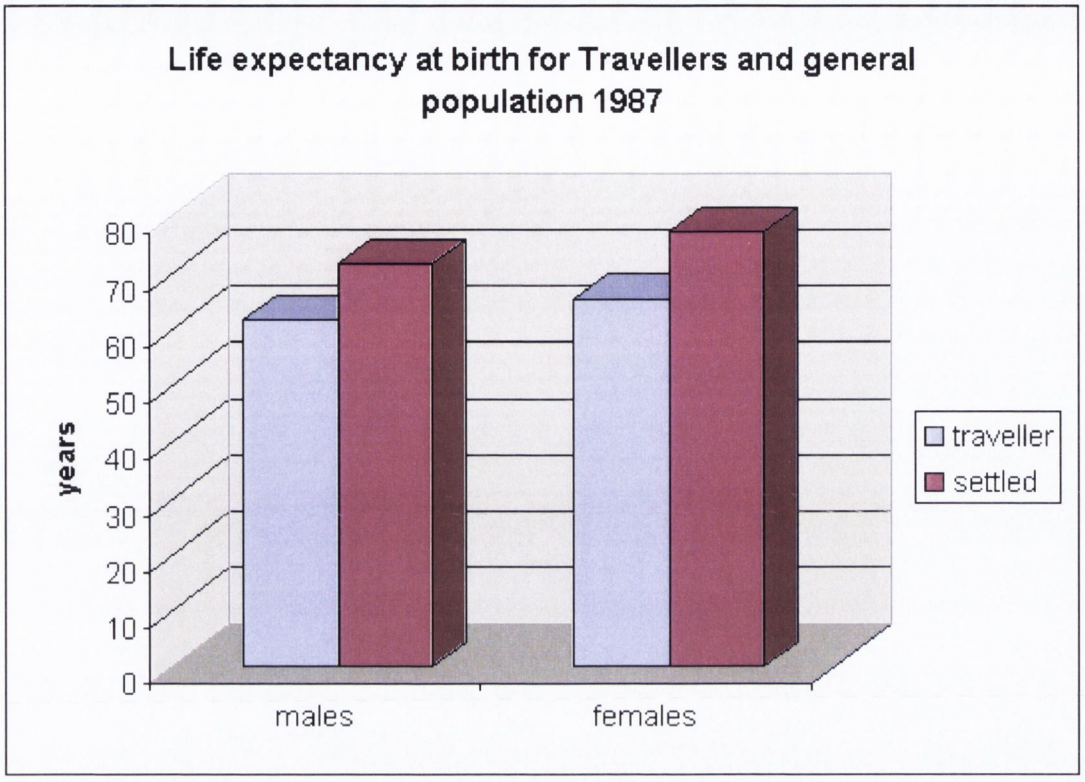
Fig 1.2 Age Distribution of General Population and Traveller Community



Key; yr, years; >, greater than; <, less than

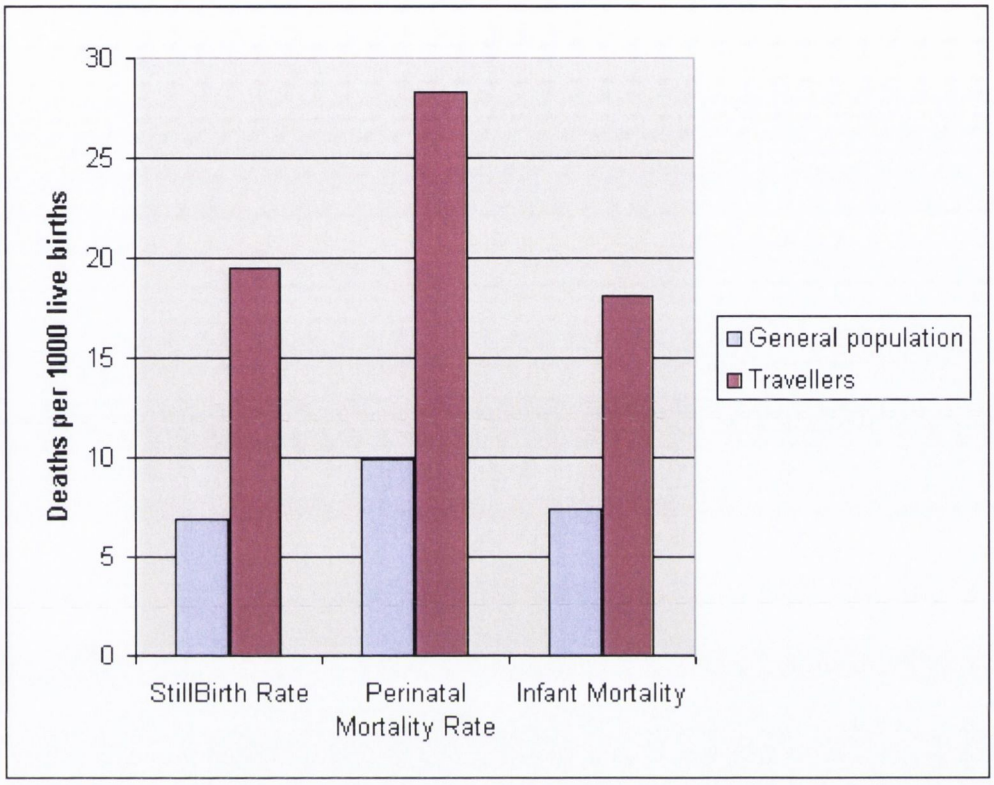
(From Traveller Health A National Strategy 2002-2005. Department of Health and Children)

Fig 1.3 Life expectancy



(From Traveller Health A National Strategy 2002-2005. Department of Health and Children)

Fig 1.4 Mortality in early life for Travellers and the General population in 1987



(From Traveller Health A National Strategy 2002-2005. Department of Health and Children)

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Chapter 2

Defining the types of inherited disease in the Traveller Community

There have been many reports written on Traveller Health (*cf* chapter 1) but little data is available about specific diseases occurring in this community. It is stated that metabolic diseases and immune disorders accounted for 3.5% of all deaths in 1987 but it is unclear what these disorders were and no further information is available. (Barry et al, 1987). A study of newborn screening records for a 25 year period, 1972-1996, found 31 cases of classical transferase-deficient galactosaemia in the Irish Travellers giving an incidence of 1:480 compared with an incidence of 1:30,000 in the non-Traveller Irish population. (Murphy et al, 1999). A previous 20 year study of the same cohort found 9 deaths in the group, 8 occurred in the first 10 years of life and 6 of these were in the Traveller community. (Badawi et al, 1996) It is suggested in the same report that several other metabolic defects including glutaric acidaemia type 1 are also common but no further details are given. The Department of Health had no specific information when asked but did send me a copy of the 1987 Barry report which looked at life expectancy and birth rates in the Traveller group. (Barry et al, 1987)

There are many problems faced by the Traveller group, including poor housing, social exclusion and lack of access to education. (Report of the Task Force on the Travelling Community 1995). All of these issues contribute to poor Traveller health and reduced uptake of health care services. It is acknowledged that 50% of all Travellers are found in Cork, Dublin, Galway and Limerick. (Barry et al, 1988) and so will presumably wish to have access to health care in these regions. If specific diseases do occur in the Travelling community it would be useful if Public Health Professionals, General Practitioners and Paediatricians, particularly in these areas, were aware that specific conditions can arise in order to institute rapid investigation and appropriate management strategies. Neonatal screening, for example, has reduced death in children with galactosaemia. (Badawi et al 1996)

The lack of readily available information regarding inherited disease in the Traveller group was an initial problem for this study. Our own haematology service had some children attending and this was a starting point. The National HLA Typing Laboratory

was another source which could be explored. The lack of a central repository of information was difficult to understand given the amount of strategic planning about Traveller health. It seemed that the way to progress the study was to ask individual Paediatricians and Haematologists about the types of disease they found and treated in children from the Traveller community and to construct our own information bank.

Paediatrician's survey

In February 2002 I wrote to all the Paediatricians in the country asking them if they had Traveller children with inherited disease attending their service. I enclosed a questionnaire to determine the nature of the diseases present in the Traveller group. (Appendix 5)

Results of Paediatrician survey.

There were 19 hospitals included in the survey; 16 regional/county hospitals and 3 tertiary referral hospitals in Dublin. (Table 2.1). Questionnaires were sent to 74 paediatricians. Forty one of these were in posts outside of Dublin and 33 in the 3 Dublin Hospitals. The response rate for those paediatricians outside of Dublin was 70.7% and for the Dublin Hospitals was 72.7%. The overall response rate was 71.6%. Three hospitals, Ballinasloe, Castlebar and Portlaoise did not respond. Clonmel and Waterford responded but had no data on the numbers of children attending their service. Eighty five percent of Paediatricians in Cork, Galway, Limerick and Dublin, where 50% of the Traveller population are said to reside ((Barry et al 1988) responded to the survey. Paediatricians in Cork said they did not have children with inherited/congenital disease whilst all of the other 3 sites acknowledged that they did have children with such disease.

Paediatricians in Limerick, Galway, Sligo and Donegal had children with Hurler syndrome attending their service. Limerick Paediatricians also had children with metabolic disease, Cystic Fibrosis and alpha1 antitrypsin deficiency. Two centres, Mullingar and Donegal, had children with Factor X deficiency. Paediatricians in the

Dublin hospitals had children with a variety of congenital/inherited diseases. General Paediatricians at The Children's Hospital, Temple Street, the National Centre for Metabolic Disease were unaware of children with congenital disease attending their clinics. It appears that all of the children going to Temple Street Hospital were attending the metabolic unit only. The Metabolic unit was unable or unwilling to release data relating to the types of disease they treat in Traveller children, stating only that Galactosaemia was more common in Traveller children than the general population. The other 2 Dublin hospitals (National Children's Hospital and Crumlin) stated that they had children with metabolic disease but also had children with liver disease, immunodeficiency, cystic fibrosis, osteogenesis impefecta and hypoadrenalism.

Data from the National Centre for Medical Genetics.

In May 2005 following discussions with the National Centre for Medical Genetics, Our Lady's Hospital for Sick Children, I was allowed access to data the centre had recently compiled about inherited disease in the Traveller group. (Prof Andrew Green Personal Communication). They had determined that there are at least 26 different autosomal recessive diseases occurring in the Traveller community. (Fig 2.1). Some of these are common e.g. Galactosaemia, Hurler syndrome, infantile Osteogenesis Imperfecta, Fanconi Anaemia and Severe combined Immunodeficiency (SCID) whilst others represent a rare genetic disorder in one particular extended Traveller family. The specific genetic mutation has been determined in only 12 of these diseases (fig 2.2) making preconception testing difficult. Their records suggested that there are 2 autosomal recessive conditions which could be classified as "haematological"; Fanconi Anaemia and SCID.

Survey of Haematologists.

In order to determine if other types of haematological disease exist in Traveller children, which may not be known to the 2 Dublin Paediatric Haematologists or Genetics Centre, a questionnaire (Appendix 6) was sent to all 9 haematologists employed in 6 of 7 health board regions (Table 2.2), the 7th region (North Eastern

Health Board) did not employ a haematologist. There were 2 Haematologists in the Western Health Board region (which includes Galway and Castlebar) but neither replied. Haematologists from all other regions replied. The haematologists serving two regions, Midlands and South Eastern Health Board were unaware of children with haematological disease in their region although a Midlands Paediatrician had reported that he was treating a child with Factor X deficiency. Haematologists from the other 3 Health Board regions reported that they had children with Fanconi Anaemia and Factor X deficiency utilising their service.

Spectrum of Inherited disease in children attending the tertiary paediatric Haematology Services.

There were 2 Paediatric Haematology centres at the time of this study, one at Our Lady's Hospital for Sick Children, Crumlin which incorporated the National Paediatric Bone Marrow Transplantation Centre and a longer established centre at the National Children's Hospital initially based at Harcourt Street and then moved to Tallaght. Both Haematology Centres supported the study.

There were 4 conditions which could be regarded as primary haematological diseases or which had significant haematological effects (Fig 2.3). The most common condition was Fanconi Anaemia. Severe Factor X deficiency was the next most common condition. Three children developed Haemophagocytic lymphohistiocytosis and one child had cytochrome B5 reductase deficiency which presented in the neonatal period with cyanosis. (Percy M et al, 2005)

Three conditions whilst not primarily haematological in nature were included because they were amenable to haemopoietic stem cell transplantation. These included a large group of children with Hurlers syndrome, six children with Carbonic Anhydrase II deficiency and 2 children with Severe Combined Immunodeficiency. (Fig 2.4).

Discussion.

There have been many Government appointed task forces and reports on the problems (social and health) experienced by the Traveller community. Nevertheless there appears to be a fundamental lack of specific information about diseases occurring in the community. There may be many reasons of this. One is undoubtedly related to the nomadic nature of Traveller life so that patients initially diagnosed in one place may not attend again for some time. Parents will, however, attend tertiary centres with their children for most, if not all, of their appointments. The second reason may be related to the vexed question of consanguinity. A study in 1986 in the Irish Midlands demonstrated that genetic disorders were concentrated in a subset of families. Seventy two percent of the marriages in these families were consanguineous and 40% were between first cousins. (Flynn, 1986). A more recent study found that there was an excess of congenital abnormalities, including metabolic disease in the traveller community and that the incidence of metabolic disease was 9.5 times greater than the population frequency. (Barry et al, 1987) It has been argued that it is not the custom of consanguineous marriage itself that is the problem but the increased risk of genetic mutation occurring because of intermarriage over successive generations. (Travellers and Consanguinity. Pavee Point Travellers' Centre publications) The Department of Health set up a working group which included representatives of Catholic Church, Travellers, Medical Profession, Genetics and Pavee Point, to advise on the issue of Consanguinity in 2000. (Chapter 9)

Data from the National Genetics Centre has identified many different types of disease in the Travellers but it appears that apart from 5 specifically identified disorders all are quite rare. Interestingly my survey identified all of the common conditions and also noted 6 others (Cystic Fibrosis, Alpha 1 anti trypsin deficiency, hypoadrenalism, Factor X deficiency, haemophagocytic lymphohistiocytosis and cytochrome B5 reductase deficiency) which had not been identified by the Genetics group.

The results of this survey demonstrate that data, where it exists, is very fragmented and there is a need for a centralised database for congenital disease in the Irish population which is readily accessible by health care professionals. Almost all of the Paediatricians and Haematologists questioned demonstrated a willingness to respond to the

questionnaires but at least some stated that their response was incomplete because they did not have their own data base.

Six regional hospitals reported children with congenital disease but nine regional centres outside Dublin did not have or had failed to recognise that Traveller children were attending their service. One of the centres which denied having children with congenital disease was the Cork region were there are about 602 Traveller families comprising about 3300 individuals (based on data from 2000; see fig 1.1). It would appear unlikely that no child with a congenital disease attends their service. In contrast to the regional centres, the 3 tertiary referral centres in Dublin all had children with congenital disease attending. This suggests that either the families around Dublin have more genetic disease, or having been diagnosed, they are happier to be followed in specialist centres and will only attend regional centres in an emergency situation. This type of attendance at the regional centres is fraught with potential danger.

The haematologists questioned were aware that 2 major haematological conditions, Fanconi anaemia and Factor X deficiency, occurred in Traveller children in their area. However one haematologist did not know that a child with Factor X deficiency was attending a paediatrician in his area and was receiving prophylactic plasma derived factor concentrate. This raises Haemovigilence issues and suggests a lack communication in that region highlighting the difficulty that can arise when one haematologist is appointed to cover an entire region.

Conclusions.

The lack of information the regional centres display about children potentially residing in their areas and attending their centres means that if these children attend in a crisis situation their needs may not be adequately met. There are 2 possible solutions to this. The first is to give "hand held" records which the family could bring with them to hospital if they needed to attend but these records may be forgotten if the child is unwell. The second and possibly most effective solution is to have a national data base which all of the regional centres could access when required. A list of recommended management strategies could be included.

This chapter sought to identify the types of disease present in the Traveller community. It appears that there are a number of diseases of particular relevance to the Haematologist. In the following chapters I will examine the more frequently occurring primary or secondary haematological diseases. I will analyse the current state of knowledge about these diseases, explore the effect of the disease in the Traveller population and evaluate current management strategies. I will finally look at HLA types in a specific section of the Traveller community, examine if there are any private haplotypes and evaluate the difficulties of finding HLA compatible donors in this group.

Fig 2.1 Data on Genetic diseases found in the Traveller Community from National Centre for Medical Genetics, OLHSC

Metabolic Disorders	Syndromes	Neurological disorders	Congenital malformations	Ocular disorders	Haematological disorders
Galactosaemia	Cohens syndrome	Freidreich Ataxia	Osteogenesis Imperfecta type II/III	Corneal hypertrophic endothelial dystrophy	Fanconi Anaemia
Glutaric Aciduria type 1	Prelingual deafness	Pontocerebellar hypoplasia type II	Fraser syndrome	Retinitis pigmentosa	Severe combined immunodeficiency syndrome (Adenosine deaminase deficiency)
Hyperprolinaemia	Byler's disease	Microcephaly	Hypoplastic left heart	Congenital anophthalmia	
Hurlers syndrome	Alopecia Universalis Congenita	Walker Warburg Syndrome	Limb hypoplasia – congenital heart disease		
I-cell disease	Congenital hypothyroidism				
Multisystem mitochrondrial disorders	Female Pseudohermaphroditism				
Carbonic Anhydrase II deficiency					

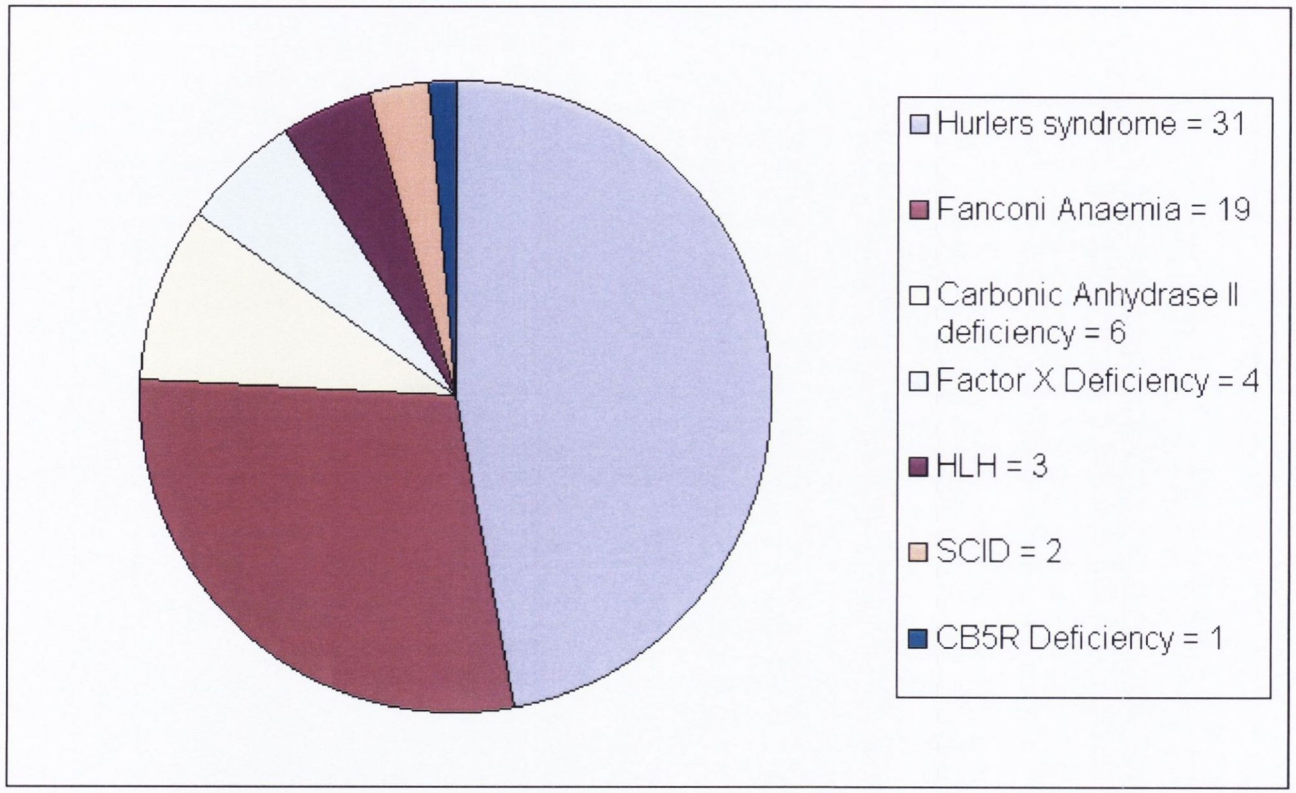
Fig 2.2 Genetic diseases found in the Traveller Community with known genetic mutations

Metabolic Disorders	**Syndromes**	**Neurological disorders**	**Haematological disorders**
Galactosaemia	Cohens syndrome	Freidreich Ataxia	Fanconi Anaemia
Hyper-prolinaemia	Byler's disease	Walker Warburg Syndrome	Severe combined immunodeficiency syndrome (Adenosine deaminase deficiency)
Hurlers syndrome	Alopecia Universalis Congenita		
Multisystem mitochrondrial disorders			
Carbonic Anhydrase II deficiency			

Fig 2.3 Primary Haematological Disease/Congenital disease amenable to Stem cell Transplant seen at Paediatric Haematology centres (OLHSC/AMNCH, Dublin)

Primary/Secondary Haematological Disease	Non-Haematological Disease amenable to stem cell transfer
Fanconi Anaemia	Hurlers Syndrome
Cytochrome B5 Reductase Deficiency	Carbonic Anhydrase II Deficiency
Factor X deficiency	
Haemophagocytic Lymphohistiocytosis	
Severe Combined Immunodeficiency	

Fig 2.4 Numbers of children with Primary or secondary Haematological Diseases attending Paediatric Haematology Services



Key: HLH, Haemophagocytic Lymphohistiocytosis; SCID, Severe Combined Immunodeficiency Syndrome; CB5R, Cytochrome B5 Reductase.

Table 2.1 Paediatricians Response to Questionnaire

Regional Hospital	**No. of questionnaires sent**	**No. of replies**	**Patients with inherited disease Y/N**	**Types of disease**
Cork	5	4	N	
Kerry	2	1	N	
Limerick	5	4	Y	Hurlers Syndrome CF Metabolic Disease α_1AT deficiency
Galway	2	2	Y	Hurlers Syndrome
Ballinasloe	2	0	-	
Castlebar	2	0	-	
Sligo	3	3	Y	Hurlers syndrome
Donegal	3	3	Y	Hurlers syndrome Factor X deficiency
Cavan	2	2	N	
Drogheda	3	2	N	
Mullingar	2	2	Y	Factor X deficiency
Kilkenny	2	2	N	
Portlaoise	2	0	-	
Clonmel	1	1	DK	
Waterford	3	1	DK	
Wexford	2	2	Y	Anopthalmia
Dublin:				
AMNCH	6	5	Y	Metabolic Disease Immuno-deficiency Osteogenesis Imperfecta
TSH	12	8	Y	Metabolic disease
OLHSC	15	11	Y	CF Liver disease Hypoadrenalism SCID

Key:
No., number; Y, yes; N, no; CF, cystic fibrosis; α_1AT, Alpha 1 anti-trypsin deficiency, FX, factor ten; DK, do not know; AMNCH, National Children's Hospital Tallaght; TSH, Temple Street Children's Hospital; OLHSC, Our Lady's Hospital for Sick Children Crumlin; SCID, Severe Combined Immunodeficiency.

Table 2.2 Haematologists response to Questionnaire

Region/Health Board Region	No. of Questionnaires sent	No. Replies	Patients with Haematological disease Y/N	Types of disease
Cork/SHB	2	2	Y	Fanconi Anaemia
Limerick/MidWestern HB	1	1	Y	Factor X Deficiency
Galway/WHB	2	0	-	
Donegal/NWHB	1	1	Y	Factor X Deficiency
Midlands	1	1	N	
Waterford/SEHB	2	1	N	

Key:

No., Number; Y, yes; N, no; SHB, Southern Health Board; HB, Health Board; WHB, Western Health Board; NWHB, North Western Health Board; SEHB, South Eastern Health Board; X, Ten.

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Chapter 3

Factor X, Factor X deficiency, and the role of prophylaxis

Introduction

Factor X

Factor X is a Vitamin K dependent blood coagulation protein which occupies a pivotal position in the coagulation process (Hertzberg M 1994). Factor X was first identified by Telfer and Hougie following evaluation of individuals with a severe haemorrhagic disorder (Telfer et al, 1956; Hougie et al, 1957). The deficient protein was called "Stuart" or "Prower" factor. Subsequently Factor X was purified and characterized from human plasma (Aronson at al, 1969; DiScipio et al, 1977).

Factor X is converted to its active form at the point of convergence of the intrinsic and extrinsic coagulation pathways. In the extrinsic pathway, Factor X is activated by Factor VII or Factor VIIa together with tissue factor. Factor X is also activated via the intrinsic pathway by Factor IXa and Factor VIIIa. Activated Factor X generated from either pathway participates in the prothrombinase complex in which Factor Xa and Factor Va activate prothrombin to thrombin in the presence of calcium and phospholipid (Fig 3.1) (Hertzberg, 1994).

Gene Structure and Regulation

The Factor X gene is 22kb long and is located on the long arm of chromosome 13q34-ter, 2.8kb downstream of the Factor VII gene (Miao et al, 1992). The genes for all of the Vitamin K dependent proteins share significant organisational and structural similarity suggesting that they have evolved from a common ancestral gene via gene duplication and exon shuffling (Pathy L, 1985). The three dimensional structure of these proteins is nearly identical with substitution of amino acids conveying specific functional properties (Hertzberg, 1994). The coding sequence of each of the Vitamin K dependent proteins is divided into eight exons, each of which encodes a specific domain

within the protein (Fig 3.2). Exon I encodes the signal peptide; Exon II, codes for the propeptide and gamma carboxyglutamic acid rich (Gla) domain. Exon III encodes a short linking segment of aromatic amino acids known as the "aromatic stack". Exons VI and V code for two regions which are homologous to epidermal growth factor (EGF). Exon 6 encodes the activation region which is at the amino-terminus of the heavy chain. Exon VII and VIII code for two serine protease (SP) domains and contain the residues of the catalytic site (His 236, Asp 282 and Ser 379) (Uprichard et al, 2002; Hertzberg, 1994).

Factor X cDNA consists of 1,474 bp, 120bp coding for the 40 amino acid pre-pro-leader sequence, 1344bp encoding the 488 amino acids of the mature protein and an unusually short 3' untranslated region of 10bp preceding the poly(A) tail (Fung et al, 1985; Leytus et al, 1984). Multiple transcription sites have been located, the majority are clustered in a region 13-33bp from the first ATG codon (Uprichard et al, 2002).

Several polymorphisms in the Factor X gene have been identified, however in contrast to Factor VII, these polymorphisms have not been found to affect Factor X levels (Uprichard et al, 2002).

Protein Structure of Factor X

Factor X is synthesized in the liver and is subsequently secreted into the blood where it circulates as a two chain inactive zymogen held together by two disulphide bonds (Fujikawa et al, 1972; Fujikawa et al, 1975). The connecting amino acids composed of Arg-Lys-Arg at residues 140 to 142 are removed by cleavage to generate a light chain (1-139) and heavy chain (143-448) (Hertzberg, 1994).

The light chain of Factor X contains 11 glutamic acid residues that are gamma-carboxylated to gamma-carboxyglutamic acid (Gla) residues. The Gla residues allow calcium dependent binding of Factor X to negatively charged phospholipid membranes (Jorgensen et al, 1987; Stenflo et al, 1977).

Post-translational modification, by glycosylation and beta hydroxylation, occurs before Factor X is fully functional. The two glycosylation sites in Factor X are located at residues Asn 181 and Asn 191 in the heavy chain and are proteolytically cleaved from FX upon activation. Beta hydroxylation occurs at Asp63 in the first EGF domain, resulting in beta-hydroxyaspartic acid (Uprichard et al, 2002). This area is believed to be a calcium binding site that may be critical in maintaining the correct conformation of FX.

Activation of Factor X

Factor X is activated to the serine protease FXa by cleavage of a single Arg194–Ile195 peptide bond in the heavy chain of the zymogen Factor X. Cleavage releases a 52 residue glycosylated peptide (Hertzberg, 1994; Uprichard et al, 2002; Tuddenham et al, 1994).

Factor X can be activated by several proteases, the two physiologically significant ones being Factor IXa and Factor VIIa. *Vipera russellii* (RVV) is a metalloenzyme that directly activates Factor X in-vitro and does not require the presence of phospholipid or calcium. In this it differs markedly from the physiological activators which are thought to form a quaternary complex on the lipid surface in-vivo. This difference has been exploited in the study of variant FX molecules to determine the regions involved in co-factor binding (Tuddenham et al, 1994).

Factor Xa Substrates

Prothrombin

Physiologically Factor Xa is the most important activator of prothrombin. The presence of the prothrombinase complex (Factor Xa, Factor Va, calcium and a suitably negatively charged phospholipid membrane) accelerates the conversion of prothrombin to thrombin 280,000 fold (Uprichard et al, 2002).

Factor V, Factor VII and Factor VIII

Factor Xa can cleave both Factors V and VIII to produce Factor Va and VIIIa. Factor VII is hydrolysed by Factor Xa to Factor VIIa in a positive feedback loop. Factor VIIa has 85 times the coagulant activity of Factor VII (Uprichard et al, 2002).

Regulation of Factor Xa Activity

Antithrombin. Factor Xa forms a stable inactive complex with antithrombin. This complex is removed from circulation by the liver.

Tissue Factor Pathway Inhibitor (TFPI). TFPI is the major inhibitor of the extrinsic pathway. Factor X binds to Factor TFPI in a one to one ratio to form a complex, which then binds tissue factor/Factor VIIa creating a quaternary inhibited complex which lacks any activity as a catalyst of the extrinsic pathway (Tuddenham et al, 1994).

Factor VIII and Factor Xa. Factor Xa has been found to inactivate Factor VIII and also takes part in its own negative feedback loop (Uprichard et al, 2002).

Methodologies for Factor X Level Assessment

There are five different assays for measuring Factor X levels. One stage PT or APTT based assays are sufficient for the diagnosis of Factor X deficiency, but due to the heterogeneous nature of congenital Factor X deficiencies, the information derived from all five assays is beneficial for detailed protein characterisation (Uprichard et al, 2002).

Immunological Assays

There are a variety of methods available to assay Factor X antigen but ELISA is most commonly used (Uprichard et al, 2002).

Functional Assays

Prothrombin time (PT) and activated partial thromboplastin time (APTT) Assays

These are the most widely used Factor X assays. They measure the ability of dilutions of standard and test plasmas to correct the PT (or APTT). Assay specificity is achieved through the use of Factor X deficient plasma, which is added to all dilutions. A number of Factor X mutants, in particular, the missense mutations, have been found to have a normal PT or APTT.

Russell Viper Venom (RVV) Assay

The venom of *Vipera Russellii* activates Factor X directly, and so will detect deficiencies of Factor X, Factor V, prothrombin and fibrinogen. However if Factor X deficient plasma is used as a substrate, then the assay will be specific for Factor X. Some Factor X missense mutations have been described with normal RVV assays (Uprichard et al, 2002).

Chromogenic Assays

These assays work on the basis that generated Factor Xa will cleave a chromogenic substrate to release a chromophoric group, which can be detected spectrophotometrically. The generated Factor Xa (and so the intensity of the colour) is proportional to the Factor X activity of the sample. Chromogenic assays are expensive and can lack specificity as the substrate may be sensitive to other enzymes (Uprichard et al, 2002).

Structure-function Relationships.

The second EGF and serine protease domains of Factor Xa are sufficient for Factor Va binding and thus play a dominant role in defining the interaction of Factor Va with Factor Xa. However, the Gla domain of Factor Xa may also be involved in Factor Va binding. If the Gla domain has been removed from Factor Xa it does not bind to

phospholipid-bound Factor Va and has a very low activity. This suggests that the second EGF and serine protease domains of Factor Xa bind Factor Va but that the Gla domain may confer additional direct, albeit weak, Factor Va binding properties to Factor X (Hertzberg, 1994).

Factor X Deficiency

Classification

The classification of Factor X deficiency is complex and based upon the results of various functional (prothrombin time, activated partial thromboplastin time and Russell Viper Venom time based assays) and immunological assays (Perry, 1997). Classical hereditary Factor X deficiency is characterized by a prolonged PT, APTT, RVVT and deficiencies in both Factor X activity and antigen. This quantitative or type 1 defect was originally described by Hougie. Other cases of Factor X deficiency have relatively normal antigen levels but the PT, APTT, RVV or chromgenic Factor X assays show variable activity. This is known as Type 2 deficiency or a qualitative defect and indicates the presence of a dysfunctional Factor X. This was originally described in the "Prower" phenotype (Telfer et al, 1956).

A complex classification of Factor X deficiency has been proposed by Fair and Edgington (Table 3.1), based on the specific response of the test FX to the various assay methodologies (Perry, 1997). It is a cumbersome classification and although it serves to illustrate the heterogeneity of Factor X deficiency it has little practical value. It is likely that a simpler and more appropriate classification system will be established when more is known about the specific FX mutations.

Congenital Factor X Deficiency

Factor X deficiency is one of the rarest inherited coagulation disorders, with a prevalence of 1:500,000 to 1:1000,000 in the general population. It is inherited in an

autosomal recessive manner and is frequently found in populations where consanguinity is common.

The prevalence of heterozygous Factor X deficiency is estimated at approximately 1:500, but is often clinically asymptomatic (Perry, 1997). Some heterozygotes do have a significant bleeding tendency and this may be due to either insufficient enzymatic activity by wild-type Factor X or inhibition of one of the reactions in the coagulation pathway by a mutant protein. It is possible that a dysfunctional Factor Xa molecule may compete with the normal Factor Xa for binding sites on Factor Va to form the prothrombinase complex. The net effect may be to reduce the formation of an active prothrombinase complex (Perry, 1997; Uprichard et al, 2002).

Clinical Aspects of FX deficiency

Severe Factor X deficient patients exhibit a moderate to severe bleeding tendency that include mucosal and umbilical bleeding, haemarthroses, post operative bleeding, deep haematomas, menorrhagia and intracranial haemorrhage (Sumer et al 1986; Peyvandi et al, 1998; Kouides et al, 2001). Antenatal intracranial haemorrhage has also been reported (Dewerchin et al, 2000; De Sousa et al, 1988; Mc Mahon et al 2002). A recent study of Factor X deficient mice demonstrated that severe deficiency can result in embryo death, which is due, at least in part, to haemorrhage. The study also demonstrated that ninety percent of the live born mice died by day 5, and the remainder had succumbed by day 20 to intra-abdominal, subcutaneous or intracranial haemorrhage (Dewerchin et al, 2000).

Patients with milder deficiency of factor X may experience easy bruising, menorrhagia or may present only following a haemostatic challenge (trauma or surgery). Some are discovered incidentally during family studies (Uprichard et al, 2002).

Pregnancy in women with severe FX deficiency

In normal pregnant women, factor X levels are reported to rise from a mean of 127% of normal activity at 12 weeks gestation to a peak of 163% at 30 weeks and thereafter to fall, returning to pre-pregnancy levels by 6 weeks following delivery (Konje et al, 1994). There is little evidence that pregnancy improves factor X levels in women with

severe factor X deficiency (Konje et al, 1994; Kumar et al 1994; Bofill et al, 1996). Pregnancy in women with severe factor X deficiency is problematic, with vaginal bleeding, retroplacental haematomata, and premature labour with fetal death all reported (Kumar et al, 1994; Konje et al, 1994). Aggressive prophylaxis during pregnancy appears of benefit for those women with a clinical history of life-long problematic bleeding (Kumar et al, 1994), whilst those with less severe symptoms may only require factor X replacement during labour and delivery (Bofill et al, 1996).

Molecular aspects of congenital factor X deficiency

Gene deletions

The earliest molecular abnormality described affecting the FX gene was that of an individual monosomic for 13q34 who was deficient in factor VII and X, whilst her brother trisomic for 13q34 had elevated levels (Perry, 1997). Bernardi et al investigated another family who had low levels of factor X activity and antigen and found that the propositus was doubly heterozygous for 2 different gene mutations. The first was a partial gene deletion which removed the 3' portion of the gene but the 2nd, inherited from her father could not be identified (Perry, 1997: Cooper et al, 1997).

A case of severe factor X deficiency arising from the inheritance of 2 non identical gene deletions was reported by Weiland et al. The first was a deletion of the entire factor X gene and the second a partial gene deletion that removed exons VII and VIII producing a type 1 defect (Perry, 1997).

The deletion of a single nucleotide has also produced severe factor X deficiency (Factor X San Antonio). This mutation (838-T) produced a frame shift and the creation of a stop codon at residue 232. The index case was also heterozygous for a missense mutation, Arg326Cys. No protein is detected, suggesting that the protein is not secreted or is rapidly broken down in the plasma (Perry, 1997). Interestingly the APTT-based assays are normal. Recently another nucleotide deletion 556-C resulting in a stop codon at residue 226 has been described (FX San Giovanni Rotondo). This is also associated with a missense mutation Lys408Asn (Uprichard et al, 2002).

Missense mutations.

The majority of mutations described in Factor X deficiency are single point mutations resulting in amino acid substitutions (missense mutations). Such mutations are of great interest in structure – function studies and a comprehensive list can be found in a recent review by Uprichard and Perry (Uprichard et al, 2002).

Mutations in the pre-propeptide and propeptide.

Factor X Nice I (Met-40Val) and FX Nice II (Pro304Ser) occurred concurrently in the proposita producing a moderate FX deficiency (antigen 7% and activity 4%). These mutations appear to cause a translation and secretion defect. Factor X Santo Domingo (Gly-20Arg) also occurs in this area. In this case the proband has a homozygous defect with antigen <5% and activity <1%. The mutation appears to prevent cleavage by the signal peptidase in the endoplasmic reticulum and so there is no further processing of the molecule (Uprichard et al, 2002).

Mutations in the Gla domains.

The loss of one of the Gla domains is likely to affect the calcium binding of the molecule which is important in its role as a coagulation protein. Factor X Ketchiken replaces a Glu residue giving Glu4Gly and results in decreased activity. Incomplete gamma carboxylation also occurs, probably leading to incomplete processing and resulting in decreased antigen levels. Factor X Voralberg is produced by 2 mutations, Glu14Lys and Glu102Lys and has a mild phenotype. Homozygotes have an activity level of <10% by PT-based assays and 25% using APTT-based assays. Factor X Frankfurt also has a lysine substitution (Glu25Lys) and a similar phenotype. It has a low factor X activity measured by PT-based assay but normal levels by APTT-based assay.

Mutations in the EGF-1 domain.

Mutations identified here affect the correct folding of the EGF domain and produce a quantitative defect (Type 1). Two mutations have been identified; Gly78Asp and Cys81Tyr. The replacement of glycine by the bulkier Asparagine is likely to affect

folding whilst the replacement of one of the 6 cysteines is likely to prevent correct formation of the 3 disulphide bridges essential for the stability of the EGF-I domain.

Mutations in the EGF-2 Domain.

Mutations in the EGF-2 domain also affect protein folding, stability and secretion and are associated with a type 1 phenotype. A Cys109Tyr mutation has been described that produces a severe phenotype due to the disruption of a disulphide bridge and a concomitant Gly94Arg and Asp95Glu mutation that disrupts folding.

Mutations in the connecting region and activation peptide.

Mutations in this region can give rise to a severe type 1 bleeding diathesis e.g. (FX Vienna {Gly204Glu}) in which both antigen and activity levels are <5%. The variant protein is produced in similar amounts to wild-type FX but FX antigen is absent from plasma suggesting in-vivo degradation by plasma proteases. Type 2 defects have also been noted in this area e.g. FX Kurayoshi caused by Arg139Ser. This mutation abolishes a cleavage site for the Arg-Lys-Arg tripeptide resulting in the failure to convert FX into a mature 2-chain form and causing an altered interaction with Va and VIIIa.

Mutations in the Catalytic Domain.

Hougie's original patient (Mr Stuart), who had a severe type 1 defect, was found to have a Val246Met missense mutation in the catalytic region. Other mutations in the catalytic domain produce only a mild bleeding tendency and some have a type 2 phenotype e.g. FX Stockton (Asp282Asn) mutation which removes aspartate from the catalytic site and has a normal FX antigen level but reduced activity. Perhaps the best characterised mutation in this region is FX Friuli. It was the first dysfunctional factor X variant to be documented in the heterozygous and homozygous state (Perry, 1997). It arises from a proline to serine substitution at residue 343 within the heavy chain. Affected individuals exhibit normal antigenic levels of factor X and near normal RVVT but prolonged PT and APTT. Substitution of Proline by serine allows the formation of a new hydrogen bond between Ser343 and Thr318 which may disturb the tertiary

structure of the region which lies beneath the substrate binding pocket of factor X. Disruption in this region may affect the catalytic potential of the molecule. (Perry, 1997)

Management of Factor X Deficiency

Factor X deficiency leads to a variable bleeding tendency and factor X levels do not always correlate well with clinical phenotype. The need for replacement therapy is often guided by the particular haemorrhagic episode.

No purified factor X concentrate is commercially available but haemostasis can be secured using fresh frozen plasma (FFP) or prothrombin complex concentrates (PCC). There have been concerns about the possible thrombogenicity of PCC (Kohler, 1999) and it has been suggested that FFP should be used in the management of minor surgical procedures and haemorrhage and that PCC be reserved for use in major surgery or life-threatening bleeds (Knight et al, 1985). The biological half life of FX is 20-40 hours (Roberts et al, 1965). The level of factor X necessary to achieve haemostasis is not known but it has been suggested that a level of 10-20% for minor bleeding episodes and 35% for surgical procedures may be adequate (Lechler, 1999; Kouides et al, 2001).

The prophylactic use of factor concentrates has been convincingly shown to significantly reduce the development of arthropathy in children with severe haemophilia A and B (Nilsson et al, 1992; Aledort et al, 1994; Liesner et al, 1996), but very little information using a similar approach in children with severe FX deficiency have been published and in those that have been, very limited follow-up has been documented. In the published studies some children have died of haemorrhage within the first year of life despite prophylaxis (Machin et al, 1980; De Sousa et al, 1988; El Kalla et al, 1993) and in one child the half-life of infused FX was found to be shorter than expected (Machin et al, 1980). A dose of 40iu/kg of PCC twice weekly or every 10 days has been suggested to provide adequate haemostasis (Sandler et al, 1992; El Kalla et al, 1993), and the placement of a central venous access device (CVAD) in younger children can facilitate this prophylactic approach. (Sandler et al, 1992). In FX deficient

patients with established arthropathy the introduction of prophylaxis may also be beneficial to reduce bleeding and improve the quality of life (Kouides et al, 2001).

In Ireland, until recently, severe factor X deficiency was only found in the Traveller community, although there are 40 recorded cases of mild factor X deficiency, exhibiting a variable bleeding tendency, within the settled community. (National Centre for Hereditary Coagulation Disorders, unpublished data). I investigated the group of children with severe factor X deficiency and also a group with mild factor X deficiency to characterise the nature of FX deficiency in this population. I evaluated the role of prophylactic replacement therapy in those with severe factor X deficiency and examined the efficacy of the dosage schedules used. I reviewed the use of Central Venous Access Devices (CVAD) to facilitate prophylaxis and assessed the complications of PCC in this population.

Patients, Materials and Methods.

The families of all children with severe Factor X deficiency were invited to participate in this study, which was undertaken between January 2001 and March 2002. The objectives of the study were explained and discussed, an information leaflet was provided and consent was obtained (Appendix 7). Parents and children with mild deficiency from both traveller and settled communities, who attended the haematology clinic during this time, were also encouraged to participate. Most of the children with mild factor deficiency had no history of haemostatic challenge and only one reported a history of easy bruising and epistaxis.

The case notes of the children with severe deficiency were analysed and data extracted using a proforma (appendix 8). Factor X levels relating to specific surgical episodes were obtained from the case notes and from the Coagulation Laboratory St James's Hospital, who performed all Factor X analysis, to determine the effectiveness of the dosing schedules and to calculate the half-life of the administered factor X.

Ten to twenty millilitres (mls) of blood was collected from each individual, depending on age. Three mls of blood was collected in EDTA for DNA analysis and dispatched by next day post to Dr James Uprichard at the Royal Free Hospital, London. The remainder was collected into 0.109molar (M) trisodium citrate anticoagulant at a ratio of 9:1. The samples were centrifuged in the Beckman Allegra 21R centrifuge at 2000g, 3333 revolutions per minute (rpm) for 20 minutes at 4°C. The plasma was removed and pooled into a 10ml polypropylene tube. Aliquots of 0.5mls plasma were decanted into vials labelled with patient name and allocated study number. The vials frozen at -80°C until assayed.

Measurement of Factor X activity (FX:C).

Factor X activity was measured by PT and APTT-based one-stage clotting assays using the ACL 9000 coagulometer (Instrumentation Laboratory Company, Lexington MA, USA). A reference plasma (Immuno) containing a defined amount of FX:C was used to construct a calibration curve and the exact sample activity was interpolated from this standard curve.

PT-based assay

Control, reference plasmas and the thromboplastin (IL Test™ PT-Fibrinogen HS plus reagent {Instrumentation Laboratory Company, Lexington MA, USA}) were reconstituted as per the manufacturers instructions and allowed stabilise for 30 minutes at room temperature. The samples for analysis were thawed in at waterbath at 37°C. The sample and control plasmas were then pipetted into the corresponding pre-labelled cups in the ACL9000 carousel beginning at position 1. The FX:C test profile was requested from the computer menu and testing commenced and the results recorded. The computer's calibration curve was reviewed to ensure that the r^2 value was greater than 0.98. (If the r^2 value is less than 0.98 the assay must be repeated). Samples are analysed neat and at 50% dilution and an average of both results determines the FX level. The results are expressed as percentage units. A control plasma (Immuno™) is measured with each test run. Those patients with results less than 5% had repeat testing using

further dilutions of CNP to construct a "low" standard curve allowing greater definition of the FX level.

APTT-based assay

Factor X activity was then measured using an APTT based methodology using IL Test™ APTT Lyophilized silica (Instrumentation Laboratory Company, Lexington MA, USA.).

Bovine cephalin was reconstituted with distilled water and allowed stabilise for 30 minutes at room temperature. The ACL9000 was loaded with the reagents (Cephalin and Calcium chloride). Dilutions of control plasmas were prepared to construct a calibration curve. The test samples and control plasmas were loaded into the corresponding cups in the same way as described for the PT-based assay and Factor X test profile is requested. Analysis of the results is undertaken in an analogous fashion.

Measurement of FX by RVVT method.

A manual one-stage Russell viper venom (RVV) assay (Diagen Ltd, Thame, UK) was performed. A standard curve was constructed using known dilutions of control normal plasma (Immuno™). The test samples were diluted 1:10, 1:20 and 1:40 in Owren's Buffered Saline. One hundred microlitres of diluted plasma sample was added to 100µl of FX deficient plasma. One hundred microlitres of a platelet substitute/RVV mixture was added and after 30 seconds 100µl 25mM $CaCl_2$ was added to achieve clot formation. The FX level is calculated from the standard curve.

Measurement of FX Antigen.

Factor X antigen measurement was undertaken by Dr James Uprichard at the Katherine Dormandy Haemophilia centre, The Royal Free Hospital, London who constructed an in-house enzyme immunoassay using microtitre plates coated with 1:1000 rabbit anti-human FX polyclonal antibody (Dako Ltd, Ely, UK). A standard curve (125 to 6.26iu/dl) was constructed with serial dilutions of pooled normal plasma in a dilution buffer. The plate was loaded with 100µl aliquots of plasma sample and controls, sealed

and incubated for 1 hour on a plate shaker (Amersham Biosciences, Buckinghamshire) at 4000 oscillations per minute at room temperature. The plates were then washed 5 times using a high salt phosphate wash buffer and 100µl 1:3000 dilution of horseradish peroxidase (HRP) conjugated rabbit anti-human FX polyclonal antibody (Dako Ltd, Ely, UK) added to each well. The plates were again sealed, incubated for 1 hour and washed. Enzymatic activity was detected by adding 100µl substrate buffer, 67%(w/v) ortho-phenylene-diamine (Sigma Chemical, Poole, UK) and 0.05%(w/v) 30% H_2O_2 to each well. The reaction was stopped using 100µl 2M H_2SO_4 per well and the optical density determined at 492 nm using a "MRX Microtitre Plate Reader" (Dynex Technologies GmbH, Denkendorf, Germany). The plate reader software calibrated a standard FX curve from the standard dilutions and the calculated the mean sample and control results from the curve.

DNA analysis

(This was performed at the Royal Free Hospital, London by Dr James Uprichard.)

Buffy coats were obtained from whole blood prepared by centrifugation at 1500g for 20minutes, and decanted into 50ml Falcon tubes. 50ml of cold cell lysis buffer was added and the mixture incubated on ice for 20 minutes. A pellet of cells was formed by centrifugation at 1000*g* for 20 minutes. The supernatant was discarded and the pellet resuspended in 1ml TE. 2ml nuclear lysis buffer was added and the mixture was gently rotated on a mechanical rotator (approximately 30 revolutions per minute) for 20 minutes. 1ml of a 25:24:1 mixture of phenol: chloroform: isoamyl alcohol was added and the sample mixed. Centrifugation at 1000*g* for 5 minutes was performed to separate the phases. The upper, aqueous, phase was removed to a clean tube using a wide-bore pipette. The sample was mixed with 1ml chloroform and centrifuged as before. The upper, aqueous phase was removed to another clean tube and 2.5 volumes 100% ethanol added. The tube was slowly inverted to precipitate the DNA. The DNA was collected onto a sealed glass Pasteur pipette and resuspended in 100µl TE pH8. The DNA samples were kept at 4°C for 72 hours to allow the DNA to dissolve.

PCR.

Amplification reactions were carried out in 100µl volumes and comprised 200ng of DNA, 100pmoles of each oligonucleotide primer, 10mM of each dNTP, 1.5mM $MgCl_2$, 1× NH_4 buffer and 2.5 units of *Taq* polymerase (all from Bioline, London, UK). A number of experiments were performed to optimise the PCR parameters. The "Hot-Start" technique was used, in which the polymerase enzyme was not added until the reaction mixture reached 94°C. Reactions were denatured at 94°C for 5 min and then 40 cycles of amplification were performed. During each cycle, the samples were denatured at 94°C for 30 seconds, followed by 30 seconds for annealing and then 40 seconds at 74°C to allow extension. On the last cycle, the extension time was increased to 10 min. For several of the exons, a "Touchdown" technique for annealing was used. In this, the annealing temperature was reduced by 0.5°C each cycle for the first ten amplification cycles. After this, a further 30 cycles of PCR was carried out, with annealing at the lowest temperature reached (5°C lower than the start temperature).

Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed to check if the PCR products were of the correct size.

Analytical grade agarose (Promega, UK) was dissolved in 0.5× TBE buffer to the appropriate concentration and heated to boiling for two minutes in a 750W microwave oven. The molten agarose was allowed to cool to approximately 50°C with stirring, before the addition of Ethidium Bromide to a final concentration of 0.5µg/ml. The gel was then poured into a horizontal casting tray with a comb of the desired size in place and allowed to set (approximately 90 minutes at room temperature). The comb and tray sealers were removed and the set gel (including casting tray was submerged in an electrophoresis tank containing 0.5× TBE. Samples were combined with 5× loading buffer (Bioline, UK) and loaded into wells, with an appropriate size marker in one well. The electrophoresis was carried out at approximately 10V/cm.

Sequence Analysis. PCR products were purified (Qiagen Purification Kit, Milan, Italy) and sequenced directly using an ABI Prism310 genetic analyser automated sequencer

(PE Applied Biosystems, Milan, Italy) with the same primers used for the amplification reactions.

Results

Clinical history of the Children with severe FX deficiency.

Case Histories

Case 1

A 15year old male, born of consanguineous parents, presented on the first day of life with umbilical cord and intracranial bleeding. His maternal grandfather had died of an intracranial bleed in early middle age. Child 1 was noted to have prolongation of both Prothrombin time (PT) and Activated Partial Thromboplastin time (APTT) and his FX level was <0.01iu/ml. He was treated with fresh frozen plasma (FFP) and was subsequently commenced on FX replacement therapy using a prothrombin complex concentrate (9A BPL) within the first month of life. Unfortunately, he developed spastic diplegia as a result of his early intracranial bleed and at 5 years of age had an uncomplicated bilateral Achilles tendon lengthening procedure undertaken using BPL 9A (70iu/kg) on an alternate day basis for 10 days to secure haemostasis. He now receives prophylactic PCC (Prothromplex ,Baxter AG Vienna), 70iu/kg weekly and has had only one bleed (right knee) since commencing prophylaxis.

Case 2

Child 2, a girl aged 5 years, is a younger sister of child 1. (Fig 3.3). She was diagnosed on the 4th day of life because of her family history. Nevertheless she had by that time developed umbilical bleeding and a haematemesis and had required PCC (BPL 9A) and red cell concentrate (RCC). Despite prophylaxis with PCC (BPL 9A, 70iu/kg) once weekly it was noted that she continued to experience significant bruising, usually 4 days following her PCC infusion which correlated with a plasma FX level below 0.05iu/ml (Fig 3.4). The frequency of prophylaxis was therefore increased to twice weekly with resolution of her bruising. Venous access had become problematic by 10 months of age and a CVAD (PortaCath; Deltac, USA) was inserted using an alternate day dosing of

PCC (40-80iu/kg) to achieve and maintain a FX level >0.5iu/ml. Although the catheter insertion was uncomplicated the line was accidentally removed within 2 weeks of its insertion. This child has had 2 further devices (PortaCath; Deltac, USA) inserted using similar PCC dosing schedules but both were subsequently removed because of persistent *Gram positive* bacterial infection. Recently a fourth device was inserted in the left arm (Passport; Deltec USA) without complication. This child has experienced 3 recent bleeds; 2 joint bleeds and one traumatic bleed over the CVAD site. The joint bleeding occurred following a reduction in prophylactic dosing. No evidence of inhibitory activity to FX has ever been documented and no further haemarthroses have occurred since the dose of PCC was increased. Objective joint assessment (Astermark et al 1999) has shown her to be normal, (Joint score 0).

Case 3

A 13 year old boy (Child 3), related to the first 2 children (Fig 3.3), presented on the 3rd day of life with umbilical bleeding, intraperitoneal bleeding and rapidly falling haemoglobin (Hb). Following resuscitation with FFP and RCC he was noted to have a prolonged PT and APTT and was subsequently diagnosed with severe FX deficiency (level<0.01iu/ml). He was commenced on prophylaxis with PCC (BPL 9A, 70iu/kg weekly). To date he has only had one bleed, (right foot) and there is no evidence of joint damage, (joint score of 0).

Case 4

Child 4, a boy aged 6 years, is one of 4 children, one of whom died of umbilical haemorrhage on day 5 of life (Fig 3.3). He also developed an umbilical ooze on the 3rd day of life which was successfully treated using FFP and was subsequently diagnosed to have severe FX deficiency (FX<0.01iu/ml). Twenty days after birth he had a severe haematemesis and required FFP and RCC. Following this episode he was commenced on prophylaxis (BPL9A, 70iu/kg) to achieve a peak level of 0.8iu/ml. Venous access became increasingly difficult, and at 2 years of age a CVAD (PortaCath; Deltec, USA) was inserted. Prior to surgery haemostasis was secured using PCC (80iu/kg) to give a FX level of 1.0iu/ml. Factor X levels were measured on a daily basis and further PCC was administered on day 2 and 6 post surgery when the FX fell below 0.5iu/ml (Fig 3.4). The CVAD remained *in situ* for 4 years and was only recently removed as it had begun to erode through the overlying skin. Removal was achieved using PCC

(Prothromplex, 80iu/kg) on the day of surgery with a further dose of (40iu/kg) on the second day following the procedure. This boy has experienced 2 bleeds whilst on prophylaxis, the first was a traumatic tongue bleed that was managed successfully giving PCC (70iu/kg) daily for 2 days, and the second, a left knee bleed, occurred because prophylaxis was omitted that week. A recent joint assessment has been normal, (Joint score = 0).

Laboratory Studies of Factor X Deficiency (Table 3.2)

Samples were obtained from 23 individuals. Two individuals (No. 22 and 23) were from the settled community the others from each of the 3 families with severe factor X deficiency. Family 1 consisted of the mother (No. 5) and 6 siblings of child 1 and 2. Child I and 2 receive prophylactic PCC as previously described. Blood sampling was performed immediately prior to receiving their scheduled prophylactic dose at the nadir of their FX level. Child 3 is from family 2. He also had levels taken prior to his weekly prophylactic dose. His 5 siblings also underwent testing. Family 3 consisted of both parents (No.18 and 21) and 2 siblings of child 4, who unfortunately was not evaluable by basic laboratory techniques as he had just received prophylactic factor X; his samples were however sent for genetic evaluation.

The PT-based FX assay is the one most commonly used to determine FX levels. Analysis of the 23 results in this study identifies that antigen, APTT and RVV results have lower mean and median results than those obtained for PT (Fig 3.5). The boxplot diagram (Fig 3.6) demonstrates this visually but also shows that the 75th percentile is similar for antigen and RVV when compared with PT. Pearson correlation analysis (Fig 3.7) demonstrates that all measures (Ag, APTT, RVV) were significantly correlated with PT but the highest correlation was seen with APTT.

All 4 methodologies give comparable low FX levels for each of the children with severe deficiency. Child 1 and 2 have some residual prophylactic FX detected but patient 3 has essentially none. These results are consistent with a severe type 1 Factor X deficiency. Classification of this defect using the Fair and Edgington system (Table 3.1) suggests that this represents a Class 8a abnormality. Clinically and using these laboratory tests

the phenotype described is similar to that found in the original Stuart family who had a catalytic domain mutation.

The diagnosis of mild factor X deficiency in all cases was based on the results derived from the PT assay, which is our standard laboratory methodology. One child from families 1 and 2 (No. 8 and 16), 2 children from family 3 (No. 19 and 20) and one child (No 23) from the settled community were designated as mildly factor X deficient by this methodology. Four of the children (No. 16, 19, 20, 23) also had reduced FX:APTT results. The fifth child (No. 8) had normal FX:APTT results but abnormal antigen results. Three of her siblings from family 1 (No. 6, 10, 11) and another member of family 2 (No.13) would also have been designated FX deficient using FX:Ag results. Individual 21, the father of child 4 had normal antigen and FX:PT results and would only be designated FX deficient on the basis of FX:APTT results. The FX:RVV correlated with a designation of FX deficiency in 6 heterozygous cases.

The results from all 4 methodologies are useful in the designation of heterozygote status and using all of these methodologies it appears that 4 members of family 1, two members of family 2, three members of family 3 and No. 22 (from the settled community) are heterozygotes for factor X deficiency.

Gene analysis

A (C→ T) mutation was noted at nucleotide 26,485 resulting in Pro382Leu (Deam S et al, 2004) This has recently been described as FX Cardiff. (Fig 3.8) This substitution obliterates a unique *Apa* I restriction enzyme in exon 8. Restriction fragment length polymorphism analysis using *Apa* I digestion demonstrates the presence of a 460bp and 418bp fragment.

Children 1, 2, 3 and 4 were found to be homozygous for this mutation. Children 6, 8, 10, 11, 13, 16, are heterozygous for the mutation and analysis of the others (No. 19, 20, 21, 23) failed.

Molecular modelling (with the permission of J. Uprichard)

MEGALIGN (DNASTAR, Madison, USA) was used to perform a multiple sequence alignment using the CLUSTAL V algorithm. Pro382 is situated in a well-conserved sequence GDSGGP that includes Ser379 (c195) of the catalytic triad. This strategic location indicates the importance of this residue in the formation of the catalytically-active folded protein structure. The serine protease fold consists of two subdomains, the first containing six β-strands B, C, D, F, G and H and the second containing six β-strands J, K, L, M, N and O (Perkins and Smith 1993). The secondary structure analysis using DSSP showed that Pro382 is situated at the end of a long loop and immediately precedes the β-strands M and N (Fig 3.9). The solvent accessibility analysis using COMPARER showed that, in the conserved loop sequence GDSGGP, all the residue sidechains occupy buried positions (0 or 1 in Fig 3.9), implying that the presence of all these residues is critical for correct folding immediately after synthesis.

Structural analysis has demonstrated that the folded protein structure will accommodate this mutation without significant distortion and in particular the structure of the catalytic triad at His236-Asp282-Ser379 is left intact. The Clinical and laboratory phenotype show a severe phenotype. It has been concluded that Pro382Leu mutation produces a disruption to the molecule's kinetic folding pathway because of the absence of the Pro residue. (Deam et al, 2004)

Discussion

The genetic abnormality identified in this group is a missense mutation in the catalytic domain as predicted using the classification devised by Fair and Edgington. It produces a severe bleeding diathesis in those homozygous for the mutation whilst those who are heterozygous are asymptomatic or have a very mild mucosal bleeding phenotype.

The results obtained from the laboratory assays demonstrated that all 4 methodologies could contribute to the diagnosis and reliance on one assay system will "miss" some heterozygotes. Our standard method for measuring FX is by PT assay system However these data suggest that FX:Ag or FX:APTT assays are more likely to diagnose FX deficiency than FX:PT (60% of cases versus 50% of cases) and the PT assay did not pick up any heterozygote who had not been diagnosed using the other assays. This suggests that FX:Ag and FX:APTT may be better methodologies to diagnose mild FX deficiency in our group. Over reliance on one methodology may limit diagnosis in susceptible families

Children with severe FX deficiency can have acute bleeding episodes ranging in severity from mild epistaxis and mucosal bleeding (Peyvandi et al, 1998) to catastrophic intracranial haemorrhage (Machin et al 1980; De Sousa et al, 1988). Intracranial haemorrhages can occur in the antenatal or postnatal period and in animal studies this type of bleeding appears to be a major cause of embryo death, at least in a mouse model (Dewerchin et al, 2000). The 4 children described here presented within the first 24-72 hours with life threatening bleeds. The introduction of primary prophylaxis with PCC has markedly changed their clinical profiles. Similar to the cases reported here, other studies have shown that haemarthroses frequently occur in severe FX deficiency and as with severe factor VIII and IX deficiency this can lead to the development of severe arthropathy (Peyvandi et al, 1998; Kouides et *al*, 2001). Haemarthroses were documented in our group when prophylaxis was omitted or the dose reduced. To date none of the children have developed evidence of "haemophilic" arthropathy demonstrating the efficacy of prophylaxis in this group.

The level of factor X required to achieve adequate haemostasis (Lechler, 1999), and the dose and frequency of factor administration required for prophylactic therapy remains to be determined. Machin and De Sousa reported that their patients succumbed to intracranial haemorrhage while on prophylaxis, prompting speculation that the efficacy or plasma half-life ($t_{1/2}$) of the PCC may not have been optimal. (Machin et al 1980; De Sousa et al, 1988) In this group a prophylactic dose of 50-70iu/kg appears to secure adequate haemostasis, but "fall-off" studies (Fig 3.4) suggest that in child 2 factor X has a $t_{1/2}$ of 16.7 hours necessitating twice weekly dosing, whereas child 4 has a $t_{1/2}$ of 27 hours and requires only weekly prophylaxis. It appears that the effectiveness of FX replacement varies from individual to individual and therefore "fall-off" studies assessing the level of FX with time should be measured in order to determine the appropriate dose and frequency of factor concentrate when prophylaxis is first commenced. It also appears that child 2 bleeds at levels below 0.05iu/ml and I suggest that in this group of patients a level >0.05iu/ml is necessary to maintain haemostasis.

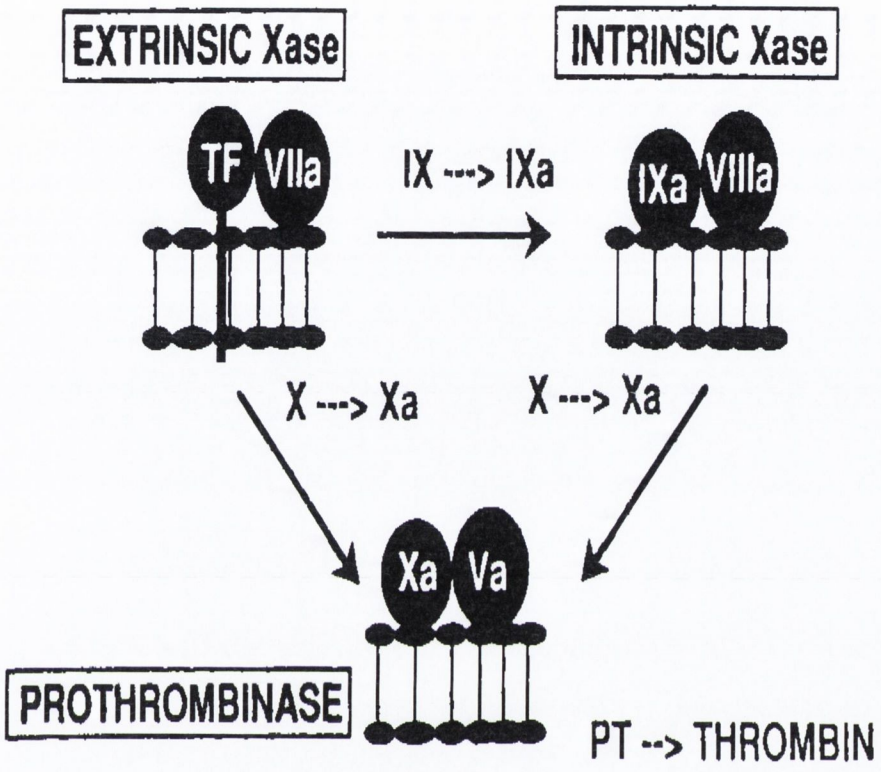
The regular administration of prophylactic factor concentrates in young children can be problematic. It has been demonstrated that the insertion of CVADs can be a useful adjunct to therapy (Leisner et al, 1995). The 2 youngest children reported here had CVADs inserted within the first 2 years of life. The surgery and post operative management was uncomplicated using a dosing schedule that maintained a FX level >0.5iu/ml for 5-7 days and CVAD removal was equally uncomplicated using a similar approach to maintain a FX level >0.5iu/ml for 2 days.

Concerns have been expressed that PCCs may be thrombogenic (Kohler, 1999). Prothrombin concentrate has been used in some of these children for over 15 years and there have been no episodes of either systemic or catheter related thrombosis. Also, the small infusion volume of lyophilised PCCs makes them the preferred FX source in treating young children with severe factor X deficiency either on demand or in a prophylactic fashion. (Sandler et al, 1992).

In conclusion, severe FX deficiency is a potentially lethal condition in early childhood and the introduction of prophylaxis with factor concentrate replacement can be life saving. The dose of prophylaxis required may vary between individuals despite having an identical FX gene mutation (Child 1 and 2), and therefore treatment should be

individualised. It appears that a primary prophylactic dose of 70iu/kg achieves optimal haemostasis in this group of young children with severe FX deficiency, although a lower dose may be equally beneficial in an older group of patients. The dose and frequency of administration should be reviewed if there is evidence of break-through bleeding while receiving prophylaxis.

Fig 3.1 Activation of Factor X



(From Perry, 1997)

Fig 3.2 Schematic diagram of Factor X molecule

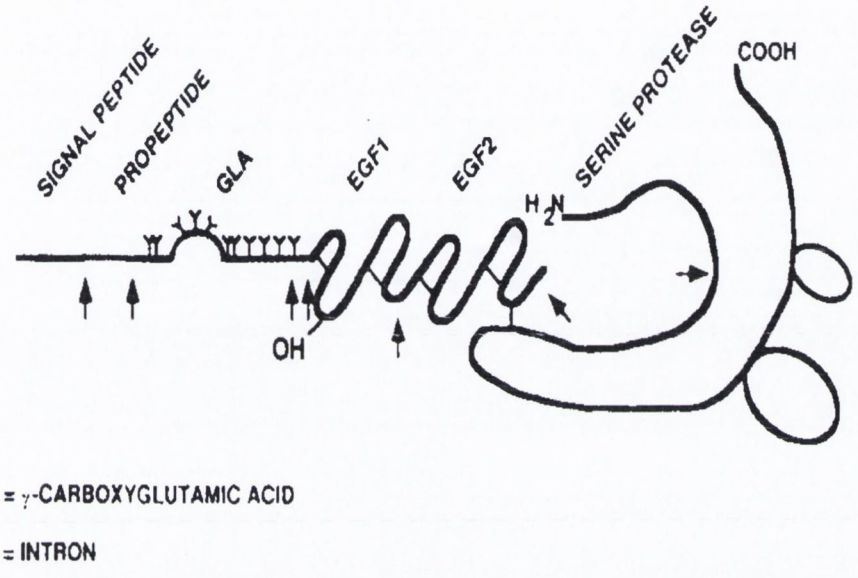


Fig. 2 Domain structure of factor X. Factor X is synthesised as a precursor molecule with a signal peptide and propeptide which are cleaved during post-translational processing within the cell. The calcium-binding Gla domain is followed by two epidermal growth factor (EGF) domains and then by the serine protease domain containing the catalytic triad.

(From Perry, 1997)

Fig 3.3 Pedigree of severe FX Kindred

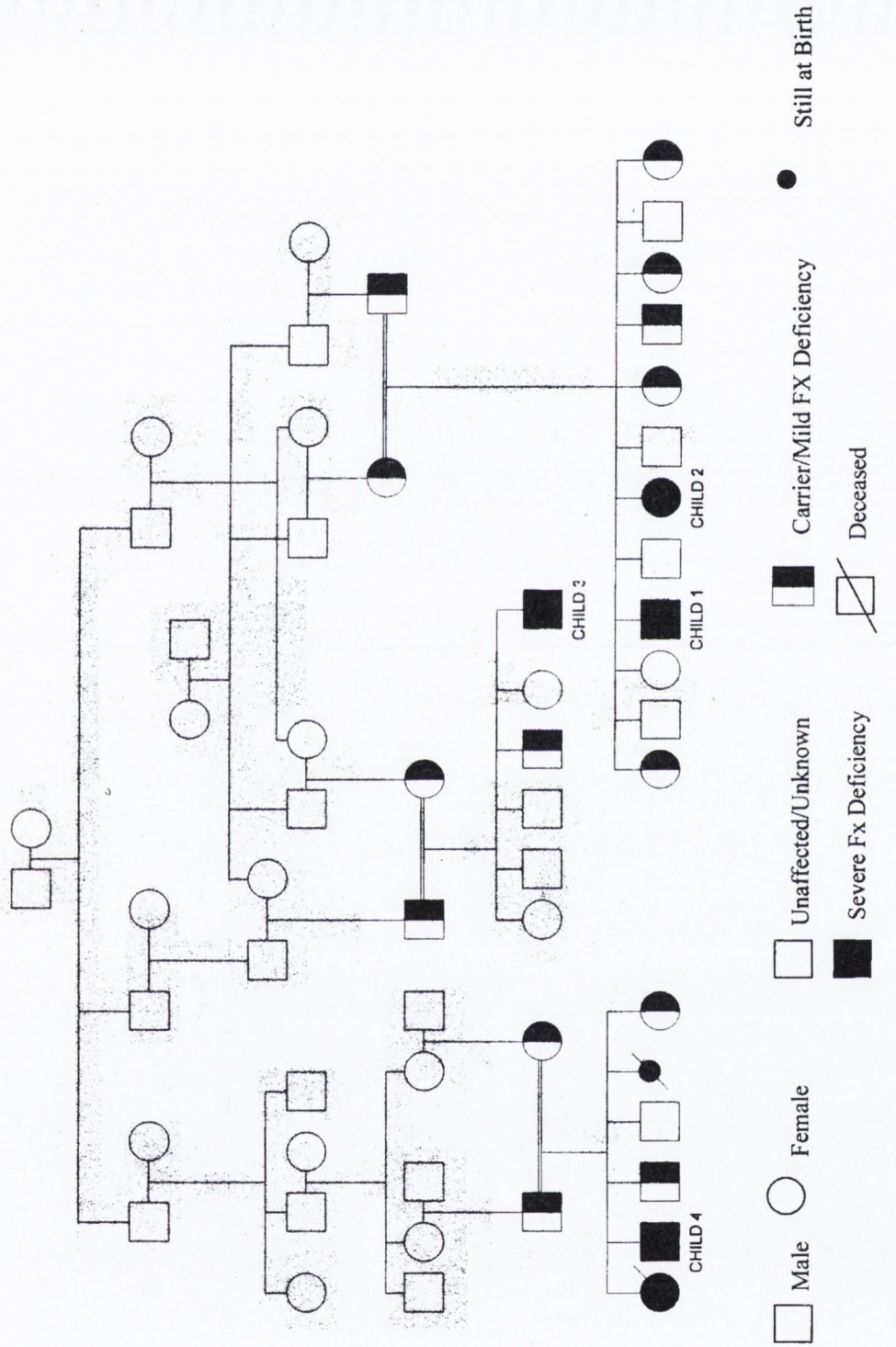


Figure 3.3 : Severe FX Traveller Pedigree

Fig 3.4 Factor X fall off data

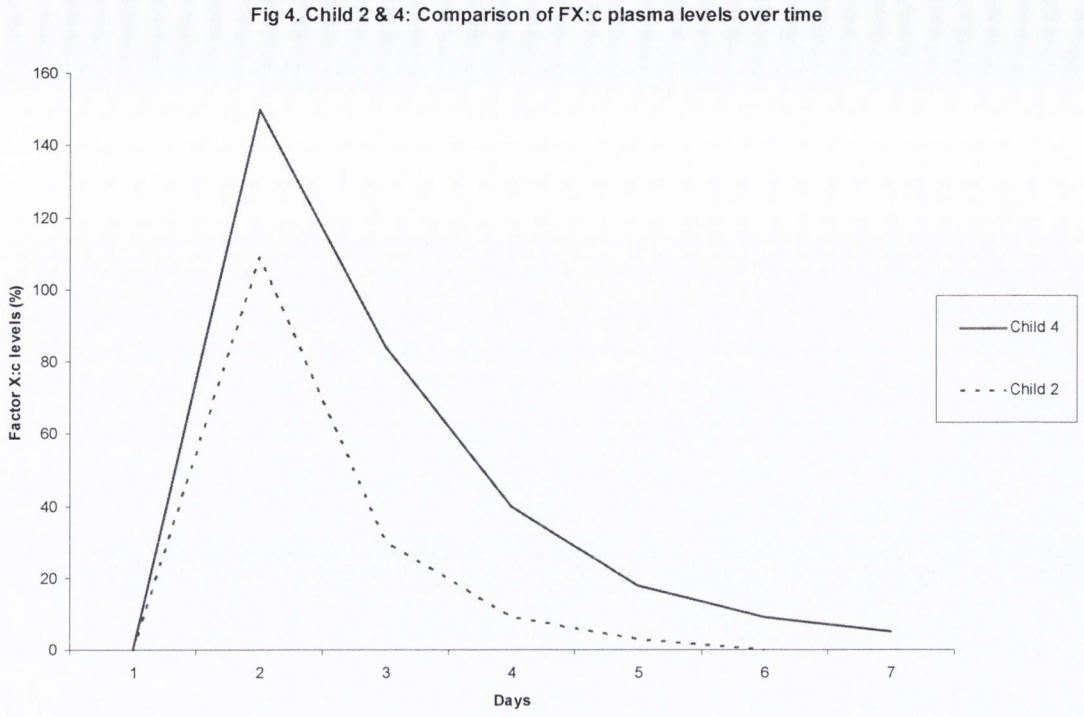


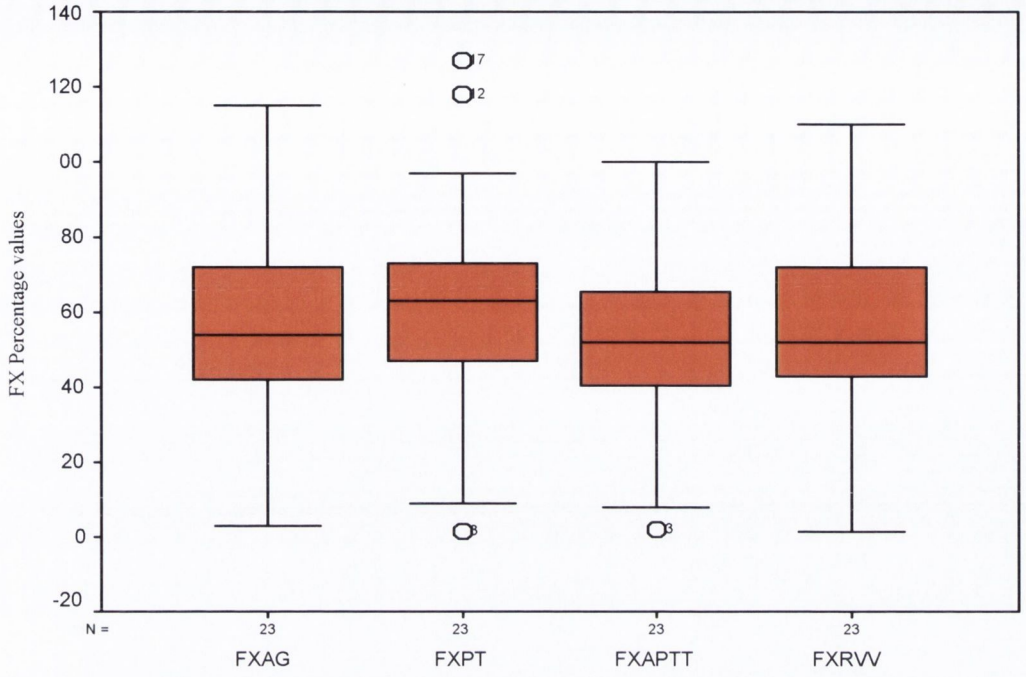
Fig 3.5. Statistical analysis of the data from all FX methologies

Statistics

		FXAG	FXPT	FXAPTT	FXRVV
N	Valid	23	23	23	23
	Missing	0	0	0	0
Mean		56.3478	60.8261	53.5652	54.4130
Median		54.0000	63.0000	52.0000	52.0000
Std. Deviation		28.00099	30.60808	24.79521	27.11786
Minimum		3.00	1.50	2.00	1.50
Maximum		115.00	127.00	100.00	110.00
Percentiles	25	42.0000	46.0000	40.0000	43.0000
	50	54.0000	63.0000	52.0000	52.0000
	75	74.0000	74.0000	66.0000	74.0000

Key; FXAg, Factor X antigen; FXPT, Factor X level measured using Prothrombin time; FXAPTT, Factor X measured using activated Partial thromboplastin time; FXRVV; Factor X measured using Russell Viper venom

Fig 3.6 Boxplot comparison of FX methodologies



Key: N, number of individuals; FXAG, Factor X antigen; FXPT, FX measured using PT based assay; FXAPTT, Factor X Measured by Activated partial thromboplastin assay; FXRVV, Factor X measured by Russell viper venom activation methodology.

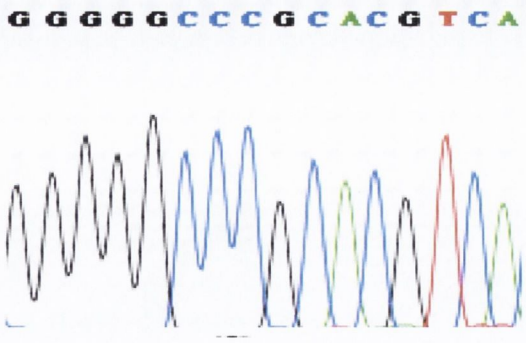
Fig 3.7 Assessment of association of PT to other methodologies

	FXPT:APTT	FXPT:Ag	FX PT:RVV
Number	23	23	23
Pearson Correlation	0.924*	0.915*	0.900*
r value	0.924	0.915	0.900

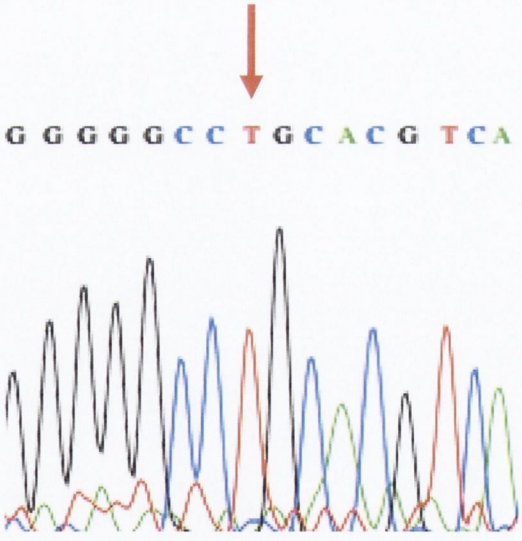
*Correlation is significant at the 0.01 level

Key; FX, Factor X; FXPT, factor X measured by PT methology; FXPT:APTT, comparison of PT based assay with values obtained using APTT methology; FXPT:Ag, comparison of PT based assay with values obtained using FX antigen; FXPT:RVV, comparison of PT based assay with values obtained using Russell viper venom.

Fig 3.8 Mutation analysis of FX deficient group



Wild-type FX exon 8



Homozygous FX
Pro382Leu

(with the permission of Dr J Uprichard)

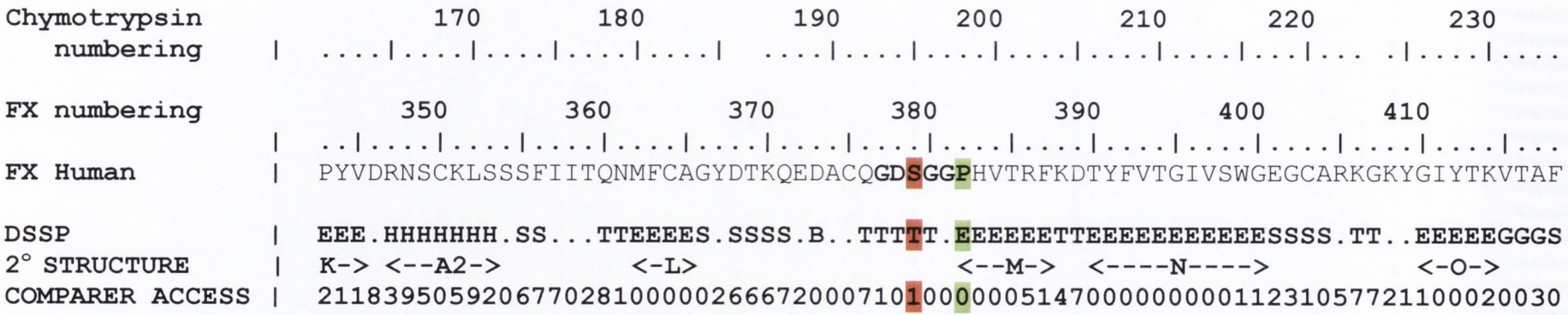


Figure 3.9 Protein sequence and structure analysis of part of the C-terminal subdomain of the serine protease domain of human FX (SWISSPROT accession code P00742). The highly conserved sequence GDSGGP is shown in bold. The DSSP output shows the consensus secondary structures found in seven experimentally observed structures of FX. The β-strands K to O (denoted by E in the DSSP output) are featured here. An α-helix (denoted by H in the DSSP output) is labelled A2. The COMPARER output shows a value (0-9) that represents the consensus solvent accessibility of each sidechain in FX. Exposed residues are identified by values of 2-9, and buried residues by values of 0 or 1.

(reproduced with the permission of Dr J Uprichard)

Table 3.1 Classification of abnormal FX molecules based upon their specific activities and antigen concentrations.

Class	RVV	PT	APTT	Ag	Diagnosis
1	N	N	N	Severe	H
2	N	N	AN	AN	A
3a	N	AN	N	Severe	H
3b	N	AN	N	AN	H
4	AN	N	AN	Severe	A*
5	N	AN	AN	AN	H
6	AN	N	N	Severe	H
7	AN	AN	AN	AN	H,A
8a	AN	AN	AN	Severe	H
8b	AN	AN	AN	AN	A,T/A
8c	AN	AN	AN	AN	H,A
8f	AN	AN†	AN†	N	H(F)

Key: RVV, Russell viper venom; PT, prothrombin time; APTT, activated thromboplastin time; Ag, Factor X antigen; N, normal; AN, abnormal; H, hereditary; A, associated with amyloidosis; T/A, transiently acquired; (F) factor X Friuli variant; A*, secondary amyloidosis; Ab†,extremely low specific activities.

(From Perry, 1997)

Table 3.2 Laboratory Results for families with factor X deficiency

Pt No.	Family No.	FX:Ag	FX:PT	FX:APTT	FX:RVV
1	1	6	9	8	6
2	1	11	14	13	10
3	2	3	1.5	2	1.5
4	3	89	96	90	70
5	1	53	68	83	75
6	1	42	55	52	50
7	1	100	97	80	100
8	1	42	49	66	55
9	1	62	64	52	59
10	1	42	63	58	60
11	1	33	54	54	50
12	2	84	118	82	80
13	2	46	64	48	43
14	2	70	72	65	52
15	2	75	74	50	50
16	2	70	46	40	30
17	3	115	127	100	110
18	3	67	64	65	85
19	3	48	44	40	40
20	3	48	41.5	40	48
21	3	62	52	45	60
22	-	74	78	58	74
23	-	54	48	41	43

FX normal range for PT-based assay: 50-100%

Key: No., number; FX, Factor X; Ag, antigen; PT, prothrombin time; APTT, activated partial thromboplastin time; RVV, Russell viper venom time

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Chapter 4

Fanconi Anaemia

Introduction

Fanconi Anaemia (FA) is an autosomal recessive condition and the name was first used in 1927 by the Swiss paediatrician Guido Fanconi to describe a familial form of aplastic anaemia occurring in three brothers with short stature, hypogonadism and skin pigmentation. Fanconi Anaemia cells are characterized by chromosomal hypersensitivity to cross linking agents producing an increase in chromosome breakage. This provides the basis for one of the diagnostic tests. Complementation analysis by cell fusion and correction of cross linker hypersensitivity has delineated at least 11 complementation groups (A, B, C, D1, D2, E, F, G, I, J, L) and currently eight genes have been cloned (FANCA, C, D1, D2, E, F, G, L) (Callen et al, 2005; Soulier et al, 2005). Recently it has been shown that FANCD1 and possibly also FANCB are actually BCRA2 (Tischkowitz et al 2003).

Epidemiology

The incidence of Fanconi Anaemia is approximately 3 per million in the general population and the heterozygote frequency is estimated at 1 in 300 in Europe and the United States. Fanconi Anaemia has been reported in many ethnic groups and founder mutations have been described in Ashkenazi Jews, who have an approximate carrier frequency of 1 in 89, and Afrikaners who are have a carrier frequency estimated at 1 in 83 (Tischkowitz et al, 2003). Recent data from the Spanish FA registry shows that there are 31 Gypsy patients with FA representing 25% of all patients registered and giving a carrier frequency of 1:64-1:70 (Callen et al, 2005). A founder mutation has also been described in the Irish population but until now the frequency has not been assessed (Faivre et al, 2000).

Clinical Features

Congenital Abnormalities

Fanconi Anaemia is a clinically heterogeneous condition and patients can have a wide variety of abnormalities. Skeletal abnormalities including radial ray defects such as hypoplasia of the thumbs and radial hypoplasia are most commonly found but congenital hip dislocation, scoliosis and vertebral anomalies can also occur. Affected subjects often have generalized skin hyperpigmentation, Café au lait spots and areas of hypopigmentation. Fanconi Anaemia is associated with altered growth both in utero and post natally. Low birth weight is common and the median height of Fanconi Anaemia patients is about the fifth centile. This can sometimes be related to growth hormone deficiency or hypothyroidism. In a recent prospective study of fifty four Fanconi Anaemia patients, 44% had a sub-normal response to growth hormone stimulation and 36% had overt or compensated hypothyroidism. Abnormalities of glucose/insulin levels were also common and the authors concluded that while short stature is an integral feature of Fanconi Anaemia, superimposed endocrinopathies may further impair growth (Wajnrajch et al, 2001). Microphthalmia, microcephaly and developmental delay are all features frequently present. Conductive deafness is also relatively common; it can be mild and may be associated with external ear malformations. Renal abnormalities are present in approximately one third of patients and include unilateral renal aplasia, renal hypoplasia, horseshoe kidneys or double ureters. In males there is a high instance of genital abnormalities such as hypogenitalia, un-descended testes and hypospadias. Infertility is usual although there have been reports of males with Fanconi Anaemia fathering children. Females may also have under-developed genitalia and uterine anomalies; older females have sparse, irregular menses but can become pregnant if not on androgen therapy.

Less common abnormalities in Fanconi Anaemia include gastro-intestinal defects such as atresia (oesophageal duodenal, jejunal), imperforate anus, tracheo-oesophageal fistulae, cardiac defects such as patent ductus arteriosus, ventricular septal defect, pulmonary stenosis, aortic stenosis and coarctation and central nervous system defects including; hydrocephalus, absent septum pellucidium and neural tube defects. Apart from isolated immune system abnormalities documented in case reports, there is no

systematic evidence that Fanconi Anaemia patients have immune deficiencies before bone marrow failure arises (Tischkowitz et al, 2003). Children diagnosed in infancy are more likely to have major characteristic anomalies. Those who are diagnosed at an older age tend to have fewer anomalies. Short stature and skin pigmentary problems become more apparent with increasing age (Alter et al, 1998).

In an attempt to diagnose those who were FA homozygotes from those who were "non-fanconi", the International Fanconi Anaemia Registry used a step wise multi-variate analysis to identify eight specific variables. One point was given for each of the following:
Microphthalmia, birth marks, genito-urinary anomalies, growth retardation, absence of radius or thumb and thrombocytopenia.
One point was subtracted for learning disabilities and other skeletal anomalies. Higher scores indicated an increased possibility of having Fanconi Anaemia (Alter et al, 1998).

Haematological Abnormalities

Haematological abnormalities are the most important clinical features of Fanconi Anaemia. It is the most common inherited bone marrow failure syndrome and the incidence of aplastic anaemia, myelodysplastic syndrome (MDS), and acute myeloid leukaemia (AML) are all greatly increased in homozygotes. The blood count is usually normal at birth. Macrocytosis is usually the first detected abnormality, followed by thrombocytopenia and neutropenia. Pancytopenia typically presents between the ages of five and ten years, with a median age of 7 years (Auerbach et al, 1991; Butturini et al, 1994). A recent review from the International Fanconi Anaemia Registry showed that there was a 90% cumulative incidence of bone marrow failure by the age of forty years. The risk of developing haematological and non-haematological malignancy also increased with advancing age and there was a 33% and 28% cumulative risk respectively by forty years of age. Males had a higher cumulative incidence of haematological malignancy than females. Univariate analysis revealed a significant early onset of bone marrow failure and poor survival for complementation group C when compared with groups A and G. However, there was no significant difference in the time to haematological or non haematological neoplasm development between these

two groups (Kutler et al, 2003b). Monosomy 5 and 7 are commonly seen in Fanconi Anaemia patients with MDS/AML (Auerbach et al, 1991). The median age at onset of leukaemia is 11.3 years, whilst the median age for solid tumours, 28.9 years, is significantly higher (Rosenberg et al, 2003).

The risk of developing a solid tumour during childhood is relatively low (but not zero) and then increases at a greater than linear rate. In one study the hazard of a solid tumour was approximately 1% per year by the age of seventeen years, 2% by age 24 years and 4% per year by age thirty years. The hazard may be close to 8% per year by the age forty. The risk of someone with Fanconi Anaemia developing a solid tumour is fifty fold higher than that of the general population. The risk is several hundred- to several thousand-fold higher for cancers of the head and neck, oesophagus, liver, vulva and cervix. Almost 1 in 3 persons with Fanconi Anaemia are expected to develop a solid tumour by the age of forty eight years compared to one per ten persons with Fanconi Anaemia who are expected to develop AML by the same age. This higher cancer incidence is not related to bone marrow transplant conditioning, and it is suggested that bone marrow transplant may further increase the risk of cancer (Rosenberg et al, 2003).

Management of the Patient with Fanconi Anaemia

The Diagnosis

Because of the heterogeneous nature of the condition, Fanconi Anaemia is difficult to diagnose by clinical features. Fanconi Anaemia cells show increased chromosome breakage. This is highly variable and overlaps with the range for normal cells. Adding DNA cross linking agents such as Mitomycin C (MMC) or Diepoxybutane (DEB) increases the number of chromosome breaks with distinct ranges for normal and Fanconi cells, so providing the basis for a diagnostic test. Chromosome breakage studies can be performed on amniotic cells, chorionic villus cells or fetal blood and therefore it can be used for pre-natal diagnosis. Although MMC/DEB testing is highly specific for Fanconi Anaemia, interpretation is complicated in cases of somatic mosaicism. Fanconi Anaemia patients have an intrinsic cell cycle disturbance

consisting of prolonged progression through and arrest within the G2 phase compartment of the cell cycle. Flow cytometry of suspected Fanconi patient cells treated with cross linkers can measure this and make the diagnosis with comparable accuracy to chromosome studies. This approach has an advantage that it is less time consuming and does not require cytogenetic expertise. It may also sometimes be helpful when there are discrepancies on DEB testing. However, it is not reliable in cases with concurrent myelodysplasia or leukaemia.

Differential Diagnosis

VATER syndrome in which vertebral defects, anal atresia, tracheo-oesophageal fistulae, renal defects and radial limb defects can arise sporadically can be mistaken for FA (Tischkowitz et al, 2003).

Thrombocytopenia–absent radii (TAR) syndrome, presents with thrombocytopenia, at birth or in the neo-natal period, and absent radii. It can be distinguished from Fanconi Anaemia because the radii are absent and the thumbs are always present whilst in Fanconi Anaemia the radii may be affected but the thumbs are always abnormal (Alder et al, 1998).

Diamond Blackfan Anaemia is characterised by defective erythroid progenitor maturation and usually presents in the first year of life with normochromic or macrocytic anaemia. Over 1/3 have congenital malformations, often involving the head (micrognathia, cleft lip), upper limbs and genitor-urinary system. It is slightly more common than Fanconi Anaemia and most cases are sporadic with evidence of autosomal dominant or less frequently recessive inheritance.

In all three conditions, MMC/DEB testing is within normal parameters allowing confident differentiation from Fanconi Anaemia.

Nijmegen breakage syndrome (NBS) is a rare autosomal recessive disorder resulting from mutations in *NBS1*, which codes for the nibrin protein. It is characterised by immune deficiency, microcephaly and hypersensitivity to ionising radiation. The

majority of patients are of slav origin and are homozygous for a single mutation in the *NBS* gene (657del5) suggesting a founder effect (Tischkowitz et al, 2003).

Somatic mosaicism (cf Chapter 5)

Somatic mosaicism may result in the reversion of the pathogenic allele to wild type in the haematopoietic cells of FA patients. Ten to twenty five percent of FA patients will have such mixed lymphocyte populations, which make interpretation of the chromosome breakage tests difficult.

The exact mechanism for somatic mosaicism can vary. One mechanism involves mitotic recombination in a bone marrow stem cell, resulting in both FA mutations on the same allele while the other allele becomes functional. Other observed mechanisms include spontaneous genotype reversion and functional correction of the mutation as a result of a secondary sequence alterations in *cis*. All may confer a selective advantage to the cell, which then repopulates the marrow and could lead to improved haematopoiesis. Some FA somatic mosaics do have a milder phenotype, but this is not always the case.

Management

Supportive Therapy

Blood and platelet transfusion have been used as interim measures for patients with bone marrow failure. Cytokines such as GCSF and GMCSF can improve haematopoiesis but should not be used in patients with a clonal cytogenetic abnormality and should be discontinued if such abnormalities develop while on therapy (Tischkowitz et al, 2003).

Androgens.

Androgen therapy for the aplastic anaemia of Fanconi Anaemia was initiated by Shahidi and Diamond in 1959, who observed improvement in the first six patients treated.

Responses usually begin with reticulocytosis, followed by a rise in haemoglobin level within one to two months. White cell counts may then increase whilst the platelet response is usually incomplete and may take six to twelve months to reach its maximum value. Almost all patients relapse with the termination of androgen therapy. Fewer than a dozen have successfully discontinued treatment, often at a time of puberty. Many ultimately become resistant to androgen therapy. Androgen responders have a projected median survival age of twenty years compared with fourteen years for the non-responders (Alter et al, 1998).

Some reports suggest that androgens alone are as effective as a combination of androgen and corticosteroids. However, the general recommendation is that combination therapy be administered as this counterbalances the anabolic properties of oxymethalone with the catabolic actions of corticosteroids (Liu, 2000).

Bone Marrow Transplantation

Bone marrow transplantation offers the only possibility of curing the bone marrow failure associated with Fanconi Anaemia and may possibly prevent the development of leukaemia. There is a risk however that chemotherapy, irradiation or both may accelerate the development of secondary malignancies. Four tongue cancers have recently been reported amongst more than fifty long term BMT survivors. This exceeds the reported 3% following bone marrow transplant for aplastic anaemia (Alter et al, 1998). In early reports, Fanconi patients undergoing bone marrow transplant had a uniformly poor outcome due to the toxicity of the preparative regimen, severe graft versus host disease (GVHD), and infection. A conditioning regimen of low dose (20mg/kg) cyclophosphamide and five gray thoraco abdominal irradiation (TAI) was then suggested based on biologic and clinical observations. Single transplantation centres, who adopted this modified conditioning regimen, with or without TAI, have reported good results for Fanconi Anaemia patients with sibling donors who did not present with leukaemia or pre-leukaemic transformation. Collected data from multiple institutions (with over 150 Fanconi Anaemia patients) yielded an overall two year survival rate of 66% (range 58% - 73%), using the lower doses of cyclophosphamide and limited field irradiation. Older age at transplantation and a low platelet count prior

to bone marrow transplant were associated with poorer survival rates, mainly as a result of increased graft failure and chronic GVHD (Liu, 2000). Recently the outcome of HLA matched related donor transplantation using the low dose regimen (cyclophosphamide 20mg/kg and TAI 500cGy) was reported on behalf of the Italian group. This showed a 19% transplant related mortality at twelve months but survival at 36 months was 81% (Dufour et al, 2001). Many patients do not have a HLA compatible sibling donor. The use of unrelated umbilical cord transplantation has also been successfully reported and may offer other possibilities for those without fully matched related donors (Yoshimasu et al, 2001). A cytoreductive regimen using Fludarabine and cyclophosphamide, followed by T cell depleted, GCSF mobilized, peripheral blood stem cell infusion has also produced minimal toxicity, early engraftment and no graft versus host disease using a two antigen mismatched related donor, suggesting yet another possible approach (Boulad et al, 2000).

All HLA-matched sibling and related donors should be screened for evidence of occult Fanconi homozygosity with thorough physical examination, complete blood evaluation including determination of red cell size and haemoglobin F level, and clastogenic chromosome stress testing prior to transplantation.

Gene Therapy

It has been shown experimentally that transfer of normal cDNA into cells from patients with Fanconi Anaemia can correct the laboratory abnormalities, suggesting that gene transfer may prevent or correct the bone marrow failure associated with Fanconi Anaemia. The major problem with this approach is in finding a suitable non-toxic vector (Croop, 2003). Following pre-clinical studies and animal safety studies, Liu et al conducted an experiment trial of *FANCC* gene therapy in three patients with Fanconi Anaemia using a retroviral vector. There was a transient improvement in bone marrow cellularity and an expansion in haemopoietic progenitors, however there was no long term haemopoietic reconstitution (Liu, 2000). More recently lentiviral vectors have been used in $FANCC^{-/-}$ and $FANCA^{-/-}$ mice to restore DEB resistance to DNA damage and normalise cell cycle abnormalities, suggesting that lentivirus gene transduction may be a better modality to use in human studies (Galimi et al, 2002).

Genetics

Until recently, eight Fanconi Anaemia complementation groups (FA-A, B, C, D1, D2, E, F, G) had been determined by somatic cell hybridization and six Fanconi Anaemia genes (FANCA, C, D2, E, F, G) had been cloned. It now appears that FANCD1 and possibly also FANCB are in fact BRCA2. Another complementation group, FA-H, has now been shown to belong to the FA-A complementation group. FANCA mutations are most prevalent, accounting for approximately two thirds of all cases of FA, FANCC and FANCG account for 25% of cases, FANCE and FANCF a further 8% and the other groups less than 1%. None of the genes cloned have any homology to each other or to any known DNA repair genes (Tischkowitz et al, 2003).

The FA genes

FANCA was cloned in 1996 and is one of the largest Fanconi Anaemia genes. More than 100 different mutations have been reported to date. Thirty percent are point mutations, 30% are 1-5 base pair microdeletions or microinsertions, and 40% are large deletions, which have removed up 31 exons from the gene. Small duplications have also been reported. The large deletions often occur at specific break points and have been shown to arise as a result of *Alu* mediated recombination (Adachi et al, 2002; Tischkowitz et al, 2003). The heterogeneity of FANCA mutations and the fact that most patients are compound heterozygotes makes diagnostic screening for mutations difficult. Mutation analysis is less complex in populations where founder mutations have been described, such as the deletion exon 12-31 mutation, which accounts for 50% of mutations in Afrikaners.

FANCA is ubiquitously expressed at low levels in the nucleus and cytoplasm of all cells, suggesting a caretaker role within the cell (Tischkowitz et al, 2003).

There is a significant genotype-phenotype correlation in FA-A: patients homozygous for FANCA null-mutations have an earlier onset of anaemia and a higher incidence of leukaemia than those with mutations producing an altered protein (Faivre et al, 2000).

Members of FA-A appear to have a relatively mild endocrine phenotype. They have more normal stature, moderate insulin resistance and no increased tendency toward primary hypothyroidism (Wajnrajch et al, 2001).

FANCC was the first gene to be cloned by functional complementation in 1992 and compared to FANCA there is a much smaller spectrum of mutations. The most frequent is the intronic mutation IVS4+4A→T which leads to an altered splice site and deletion of exon 4. This is particularly prevalent in Ashkenazi Jews with a carrier frequency of 1 in 89 and is characterized by a severe phenotype with multiple congenital abnormalities and early onset of bone marrow failure. The same mutation is also the most prevalent FANCC in the Japanese population (thought to be the result of separate founder mutation), but the phenotype is less severe compared to Ashkenazis, suggesting that race specific modifying genes or other environmental factors influence phenotype severity. (Futaki et al, 2000). Other common mutation sites are found in exon 1 and exon 14. FANCC is ubiquitously expressed and the protein has been localised to both cytoplasm and nucleus.

FANCD1 has very recently been shown to be BRCA2 by sequencing by BRCA2 gene in cell lines from FANCD1 patients who were subsequently found to be compound heterozygotes. BRCA2-/- cells exhibit many of the features of Fanconi Anaemia cells such as chromosome instability and hypersensitivity to crosslinking agents but null mice are not viable and no humans with biallelic germline BRCA2 mutations have been reported before, suggesting that this is not normally compatible with life in man.

FANCD2 is the most recently cloned gene and although it accounts for only a small number of Fanconi Anaemia families, it plays a key role in the Fanconi Anaemia pathway. The FANCD2 protein exists in two forms, FANCD2 short (S) and FANCD2 long (L), the long version being formed by the addition of an ubiquitin molecule to a lysine residue at position 561, a process known as monoubiquitation. Once FANCD2L has undergone monoubiquitation it co-localises with BRCA1. Fanconi Anaemia cells deficient for FANCD2 are sensitive to MMC but also have an increased sensitivity to ionizing radiation similar to that seen in Ataxia- telangiectasia (AT). When normal cells are exposed to ionizing radiation, the AT protein product, ATM, phosphorylates FANCD2 at serine 222, activating a checkpoint response, a mechanism which is distinct

and independent of MMC induced monoubiquitination. FANCD2 therefore provides a link between the FA protein complex and other pathways implicated in DNA repair by functioning at the intersection of two pathways, one involving MMC inactivation by the FA protein complex and the other involving ionizing radiation inactivation by ATM. (Fig 4.1)

The **FANCE** and **FANCF** genes were both cloned by functional complementation and between them account for less than 10% of all Fanconi Anaemia families. FANCG is identical to XRCC9, a human gene that corrects chromosomal instability and mutagen sensitivities in Chinese hamster ovary UV40 cells. All types of mutations except large deletions have been reported in FANCG (Tischkowitz et al, 2003).

In a genotype-phenotype study of 245 Fanconi Anaemia patients from all complementation groups, a more severe cytopenia and higher incidences of leukaemia was detected in the FAG group. There was an earlier onset of anaemia and a higher incidence of leukaemia in those patients with FANCA null mutations, and a reduced incidence of somatic abnormalities in the FAC group excluding the IVS4+4A-T mutation. Patients with FACG mutations and FANCA null mutations appear to constitute a particularly high risk group (Faivre et al, 2000).

Phenotype similarities between the different FA complementation groups suggest that the FA protein products interact in a common pathway. (Fig 4.1) Current evidence suggests that a core complex composed of FANCA, C, E, F and G leads to activation of FANCD2S to FANCD2L by monoubiquitination and FANCD2L then interacts with BRCA1. FANCD2 also interacts independently with ATM activating the S phase checkpoint response. (Fig 4.1) Recently it has been suggested that the deubiquitinating enzyme USP1 deubiquitinates FANCD2 when the cells exit S phase and so recycle FANCD2 (Nijman et al, 2005). Fanconi Anaemia may be linked at a molecular level to other DNA damage repair disorders because the BRCA1 associated genome surveillance complex (BASC) includes DNA damage repair proteins such as ATM, NBS1, MRE11, BLM and MSH2 (these genes are mutated in ataxia-telangiectasia, Nijmegen breakage syndrome, AT-like disorder, Bloom's Syndrome and HNPCC respectively). The discovery that FANCD1 and possibly FANCB are in fact BRCA2

also indicates that the pathways involved in breast cancer susceptibility and Fanconi Anaemia are interconnected at more than one level (Tischkowitz et al, 2003).

The FA Functional Pathway

DNA Repair in the FA Cell

The exact mechanisms that lead to disruptions to DNA repair in FA cells remains disputed. Repair of interstrand cross links, induced by agents such as DEB and MMC, necessitates incision of DNA but if both strands of DNA are damaged simultaneously there is no available undamaged template. Double stranded breaks can be repaired by non-homologous end joining where two DNA ends are ligated together at regions of little or no homology. Studies where plasmid extracts have been induced into FANCC mutated cells have shown a reduced fidelity of non-homologous repair of blunt ended double stranded breaks compared to normal cells leading to a deletion prone phenotype which can be corrected by complementing the cells with wild type FANCC. Similar error prone phenotypes have been described in FANCD1 defective cells and also in other chromosome instability syndromes such as Bloom's Syndrome, ataxia-telangiectasia and Werners Syndrome.

In addition to abnormalities in the non-homologous end joining region it is possible that the detection of DNA damage in the Fanconi Anaemia cell is also impaired. There is evidence that the S phase cell cycle checkpoints are altered in that interstrand cross links did not slow down or arrest Fanconi cells in the S phase, with arrest instead occurring at the G2 checkpoint after completion of replication. These studies indicate that FA proteins play an important role in DNA repair, which may be either direct involvement in repair of the DNA itself and/or in DNA damage signalling pathways mediating cell cycle checkpoints.

Other Functions of FA proteins

In addition to defective DNA repair, a number of other possible causes of the abnormalities found in Fanconi Anaemia patients have been postulated.

Cell Cycle Control

G2/M transition in the cell cycle is prolonged in Fanconi Anaemia cells and this is further increased if cells are exposed to cross linking agents or high oxygen concentrations. The G2/M checkpoint is associated with surveillance of the genome and repair of damage before progression to mitosis. This observation could be explained by the fact that FA cells fail to repair DNA damage efficiently and therefore arrest at G2 (Tischkowitz et al, 2003; Liu, 2000).

Oxygen Sensitivity

Fanconi Anaemia cells exhibit increased oxygen sensitivity and chromosome breakage is reduced in the presence of low ambient oxygen concentrations. Many DNA cross linking agents produce reactive oxygen species and it is possible that the sensitivity of FA cells to cross linkers is the result of an impaired ability to counteract reactive oxygen species. Fanconi Anaemia cells grow very poorly at ambient (20%) oxygen tension but grow well at reduced (5%) oxygen tension (Liu, 2000).

Apoptosis and telomere maintenance

Various studies have shown that apoptosis is abnormally regulated in FA cells although the exact nature of the abnormality remains disputed. Increased apoptosis may be related to the inability of FA cells to repair damage or to defective FA protein interactions with other proteins in the apoptosis pathways. Accelerated telomere shortening has been detected in Fanconi Anaemia, but is also found in other forms of aplastic anaemia and in MDS. This could be explained by a haemopoietic stem cell undergoing a higher than normal number of cell divisions to generate mature cells, but, a recent study has shown that there is also a higher rate of telomeric breakage in Fanconi Anaemia cells, suggesting a defect in telomere maintenance. It has been suggested that the individual annual telomere-shortening rate (IATSR) in peripheral blood mononuclear cells correlates with the development of severe aplastic anaemia. Spontaneous apoptosis is highest in patients with high IATSR (greater than 200 BP per year), a high IATSR was also associated with an increased frequency of malignancy. It

is suggested that this biological parameter could be used as a prognostic factor in the evolution of Fanconi Anaemia (Li et al, 2003).

Haemopoiesis

In vitro bone marrow culture assays have shown defective haemopoiesis in Fanconi Anaemia and FA cells show altered levels of certain growth factors such as reduced IL-6, GM-CSF, IL-1 beta and increased TNF-alpha (Stark et al, 1993; Liu, 2000; Tischkowitz et al, 2003). These may affect haemopoiesis by altering the bone marrow microenvironment, leading to deregulation of cellular homeostasis, differentiation and response to stress.

Haematopoietic tissue is one of the tissues most sensitive to DNA damage caused by radiation or cytotoxic therapy but while other DNA repair disorders such as Xeroderma Pigmentosum or Ataxia Telangiectasia have an increased risk of developing certain malignancies, they do not have the same incidence of bone marrow failure seen in Fanconi Anaemia. It therefore seems likely that Fanconi Anaemia proteins play a specific critical role in the maintenance of haematopoietic stem cells and that a combination of the different functions of the Fanconi Anaemia pathway are implicated. The initial decline in haematopoietic stem cells leading to bone marrow failure could be the result of defective telomere maintenance and high rates of apoptosis. Selective pressures may then promote the development of mutant clones because of the altered cytokine signalling environment and genome instability, leading to MDS and ultimately to AML.

Endocrinopathy

A recent study has demonstrated endocrine abnormalities in 81% of FA patients studied. These included short stature, growth hormone deficiency, hypothyroidism, glucose intolerance, hyperinsulinism and/or overt diabetes mellitus. Patients with FA-A and FA-G were relatively taller than the whole FA group but smaller than the general population whilst those with FA-C were smallest. There was abnormal endogenous growth hormone secretion in all patients suggesting an underlying hypothalamic-pituitary dysfunction. It is also suggested that these endocrinopathies may be a secondary effect,

mediated by TNF alpha and IL6, as both TNF alpha and IL6 deregulation have been associated with insulin resistance, as well as abnormal thyroid function, decreased thyroglobulin, thyroid peroxidase and reduced serum T3 levels (Wajnrajch MP et al, 2001).

The majority of cases of FA found in the Irish population are within the Irish Traveller group. I defined the number of children with this condition within the traveller group and the nature of their genetic mutation. I characterised the natural history of FA within this group and thus the natural history of this genetic mutation. I have explored the role of BMT and the outcome of FA following BMT.

Patients Materials and Methods

Patients

Data was obtained and collated from 3 sources; the HLA typing requests from the records of the National Blood service, the records of the National Paediatric Bone Marrow Transplantation centre, Our Lady's Hospital for Sick Children, Crumlin and the National Children's Hospital (NCH) formerly at Harcourt Street, now located at AMNCH Tallaght. Our Lady's Hospital for Sick Children, Crumlin and National Children's Hospital were the 2 tertiary paediatric Haematology centres in Ireland to which children with FA could be referred for management. Information was also obtained from the families of children with FA, some of whom were aware of the extended family history of FA. No information about the children with FA was forthcoming from either general paediatricians or haematologists in the regional hospitals. (*cf* chapter 2).

Initial assessment.

The children underwent physical examination, and had height, weight, and head circumference plotted on standard Tanner growth charts at their initial visit to the

tertiary centre. They all had radiological examination of kidneys and some had a full skeletal survey whilst in others radiological examination was limited to upper limbs. All had full blood count estimation (FBC) and some bone marrow aspirate (BMA) and biopsy with cytogenetic examination.

The diagnosis of FA was confirmed by the study of chromosomal breakage, induced by diepoxybutane, in the peripheral blood lymphocytes. This was performed at the Regional Cytogenetic Service Bristol UK, using a methodology described in Current protocols in Human Genetics (Auerbach, 1994). Some of the group also had molecular analysis and complementation studies undertaken at Professor Christopher Mathew's Laboratory, Guys Hospital, London.

Management.

The children, their immediate families and in some cases extended families (Aunts, uncles, cousins) had 10 mls of blood taken in an acid citrate dextrose solution for HLA typing (See chapter 8) to determine the availability of a suitable Bone Marrow donor.

The children were monitored at a minimum of 6 monthly intervals for the onset of bone marrow failure or malignancy. Following the development of Pancytopenia (defined as Hb<10g/dl, platelets <100x10^9/l and or neutropenia <1.0 x10^9/l) the children were reviewed more frequently. Specific blood component transfusion was initiated for symptoms and if the platelet count was <20 x10^9/l (since 2000 this trigger has been reduced to <10 x10^9/l) or Hb <8g/dl. Some children received androgen therapy (Oxymethalone 2-4mg/day) in an attempt to improve bone marrow function and to reduce blood transfusion requirements

From 1988 – 1999 blood components were CMV negative, irradiated and transfused using a leukocyte filter. More recently, following changes to blood component manufacture at the IBTS, bed side leukocyte filtering is no longer undertaken. Children with bone marrow failure syndromes now have red cell phenotyping undertaken prior to their first blood transfusion and transfused red cells are also Rhesus and Kell red cell antigen matched.

Bone Marrow Transplantation

All of the transplanted children were conditioned with 120mg/kg total dose cyclophosphamide over 4 days prior to bone marrow transplant. Acyclovir (500mg/m2 tds), and itraconazole (5mg/kg/day) were given as prophylaxis against viral and fungal infection. Ursodeoxycholic acid (10mg/kg/day) was administered from day -6 to day +30 as prophylaxis against veno-occlusive disease of liver. Cyclosporin A, (1.5mg/kg iv from day -1), and Methotrexate (10mg.m^2 on day +3 and +6) were given as graft versus host prophylaxis. The dose of cyclosporin was adjusted to maintain a level of 100-300. Five hundred milligrams per kilogram intravenous immunoglobulin was administered weekly for 4 weeks then three weekly for 3 months post transplant. Cotrimoxazole or pentamidine was commenced when the neutrophil count had exceeded 1.0 x10^9/l following transplant to aid Pneumocystis Carinii prevention.

Engraftment was determined by restoration of normal haematopoiesis and by chimerism studies.

The children have twice weekly follow-up initially post-transplant and the period of review is slowly extended if they remain well. Finally they are reviewed on a 6 monthly basis to monitor bone marrow function, growth, development and the onset of any symptoms which may indicate the development of malignancy.

Results.

Nineteen children (11 girls and 8 boys) were found to have been diagnosed with Fanconi Anaemia from 1982 – 2001 when data from all 3 sources was collated. The names of five children were found in the records of the National Blood Service but no matching hospital data was discovered, although 3 children had HLA compatible sibling donors and might have been expected to undergo BMT. The outcome for 3 of the children was uncovered incidentally from the family histories of other children subsequently diagnosed with FA.

Data was available for the remaining 14 children. All the affected children are related. Three families had 2 affected siblings and a fourth family had 3 affected siblings. There were 7 boys and 7 girls diagnosed between 1 month and 7 years of age (median 28 months). The reasons for diagnosis were a combination of family history (16 cases), physical appearance (3 cases), short stature (3 cases), failure to thrive (3 cases) and bruising due to thrombocytopenia (4 cases) (Table 4.1).

Five children have Fanconi mosaicism; clastogenic stress testing shows 50% normal cells and 50% cells with breakage. Two of these children (No. 12 and 14) were diagnosed incidentally when they presented with aggressive Haemophagocytic lymphohistiocytosis (HLH) and another because she was a sibling of one of these children. The other 2 children with mosaicism were diagnosed because of typical physical appearance and family history. One child (no. 10) who had a family history of FA and failure to thrive, was diagnosed by analysis of tissue fibroblasts following a normal DEB test.

Physical appearance (table 4.2)

All the children had abnormal pigmentation, either hyperpigmentation, café au lait spots or areas of hypopigmentation. The majority had short stature, microcephaly, characteristic "bird-like" facies and thumb abnormalities (rudimentary or absent thumbs and hyperextensibility of the joints). Five children had ear anomalies which consisted of abnormally shaped ears and/or auditory canal stenosis. Four children had renal abnormalities consisting of renal agenesis, horseshoe kidney or hydronephrosis. Four children also had cardiac abnormalities, either a ventricular septal defect, patent foramen ovale or patent ductus arteriosus. One boy had hypospadias and another undescended testes. None of the girls had genital anomalies. One child with FA mosaicism had essentially normal appearance.

Bone Marrow Failure. (Table 4.3)

Four children who were diagnosed within the first year of life had normal blood counts at presentation. One, child 10, is discussed below. Two of the others, (child 6 and 7) are

still under 36 months and their blood counts remain normal. The last child, child 3, developed bone marrow failure at 64 months (fig 4.3) and became transfusion dependant at 66 months (table 4.3). Five children were referred for assessment at 20-65 months (median 44 months).

Child 11 was already mildly thrombocytopenic at presentation and rapidly developed bone marrow failure (fig 4.2) becoming red cell and platelet dependant by 38 months. He developed monosomy 7/AML at 95 months and finally died of his disease 4 months later. Child 2 became platelet transfusion dependant by 44 months of age and red cell dependant by 55 months. Two others (child 4 and 1) already had evidence of bone marrow failure when referred and became transfusion dependant at 62 and 75 months respectively. They were subsequently commenced on androgens. A total of five children received androgens (oxymethalone) in an attempt to halt their bone marrow failure or to reduce their transfusion requirement. (Table 4.3) They commenced androgens between 43-89 months, median 72 months. It was unsuccessful in 3 cases (child 2, 4 and 11). Child 1's red cell transfusion requirement reduced to one transfusion every 3 months and child 3 did not become platelet dependant prior to BMT.

To date, seven children have become transfusion dependant and 5 of these became platelet dependant about 6 months prior to becoming red cell dependant.

Fanconi Mosaicism

Two children (child 12 and 14), presented with pancytopenia, caused by haemophagocytic lymphocytic histiocytosis (HLH). One of these children had complete normalisation of his blood counts following resolution of this disease and at the age of 84 months there has been no progression to bone marrow failure. The other child developed fulminant HLH and an associated bone marrow failure but did not survive (chapter 5). Child 13, a sibling of child 12 is aged 141 months and has normal blood counts.

One of the other children with FA mosaicism (child 5) had normal blood counts for the first 60 months of life and then developed rapidly progressive bone marrow failure over the next 30 months (fig 4.2). She finally became red cell dependant at 104 months (Table 4.3). Child 9, another child with FA mosaicism, behaved in a manner more typical of those with Fanconi syndrome and had developed significant bone marrow failure by 52 months (Table 4.1). She was transfusion dependant by 58 months (Table 4.3).

Child 10 was diagnosed using skin fibroblasts because clastogenic stress testing of peripheral blood lymphocytes failed to demonstrate any abnormality. She is now 48 months of age and her blood count remains normal.

Other complications (table 4.3)

There is some evidence that 19 children have had FA in the past 25 years. Seven children with FA are reported to have died of their disease, however hospital records are available for only 2 of these, information on the others was obtained from related families with FA. Child 11 finally developed monosomy 7/AML at 95 months of age and died 4 months later. Child 12 who had an Epstein Barr driven HLH developed a gastrointestinal lymphoproliferative disease at 35 months. This was fully resected and he remains well and in remission 51 months later. Child 14 died of fulminant HLH (*cf* Chapter 5).

Outcome of bone marrow transplantation. (Table 4.4)

Six children, 3 boys and 3 girls received bone marrow transplants since 1990. Two had sibling donors and the other 4 received donations from other family members e.g. cousins. (CF Chapter 8). Transplantation was undertaken between 73 and 144 months of age, median 80 months. All of the children had been transfusion dependant for 6-35 months, median 20 months, prior to transplantation. Four children received androgens for 9-45 months, median 15 months, prior to transplantation.

All of the children received the same preparative and prophylaxis regimens. Three children had no problems during transplant whilst the other 3 developed either haemorrhagic cystitis which cleared with hydration, veno-occlusive disease of the liver or acute GVHD. All of the children are alive and full donor chimeras between 11 and 72 months, median 43months post transplant.

Genetic mutation.

All of these children are related (Fig 4.3) and 5 have had gene analysis undertaken at Professor Christopher Mathew's research laboratory, Guy's Hospital, London. This has demonstrated a deletion of exons 11-14 in the FANCA gene in all of those tested.

Discussion.

Fanconi Anaemia is a rare cause of bone marrow failure found only sporadically in the Irish population. It is however the commonest cause of bone marrow failure on the Traveller population, with a prevalence based on my data of 1:1250. All seven of the children on whom DNA analysis was undertaken have FA-A. The specific mutation del11-14 is an apparent founder mutation in this population (Faivre et al, 2000). It is likely that this is the genetic defect in all of the children because of their close family relationships.

Although records suggest that 19 children have been diagnosed with FA in the past 20 years, no further data could be obtained on 5 children who were diagnosed in the 1980s which demonstrates yet again the problems associated with inadequate data keeping. The outcome in these children was elucidated from family histories of children diagnosed at a later date.

Data from 14 children was evaluable. Four children, diagnosed because of physical appearance and strong family history, were referred within the first year of life to tertiary haematology centres and had normal haematological values at presentation. Early referral permits appropriate treatment planning including HLA donor search and

careful regular monitoring of bone marrow function. In contrast seven others were only referred because of worsening bone marrow failure, although in most cases the diagnosis had been made at an early age. Two of the children were only diagnosed because of fulminant HLH and their progress will be discussed in the following chapter. This pattern of referral and diagnosis is not unusual. Data from IFAR found that the majority of FA patients were not diagnosed until after the onset of haematological abnormalities, although they did have congenital abnormalities (Giampietro et al, 1993).

All the children had some characteristic features and the majority (70-80%) had the classical phenotype of microcephaly and radial ray defects. This differs from the proportion of somatic abnormalities previously reported for group A FA, which suggests that 75% will have skin abnormalities and 53 and 49% head and radial ray defects respectively (Faivre et al 2000). Thirty three percent of our group had auditory abnormalities compared with 11% from the EUFAR and 26% of our group had cardiac anomalies compared with 9.4% in EUFAR (Faivre et al, 2000). In contrast the numbers of children with genital and renal anomalies was remarkably similar. Ten of our group (70%) had short stature (height and weight $<3^{rd}$ centile) and in the case of 5 children this was one of their presenting features. Previous studies have demonstrated growth retardation in this group. In Faivres study 60% of FA patients had growth retardation. It is suggested that growth is only mildly retarded in FA-A at -1.55±0.45SD compared to -2.35±0.28SD for all groups, however our group had height and weight below the 3^{rd} centile suggesting a more severe growth restriction (Wajnrajch et al, 2001). These data suggest that this large deletion in the FACCA gene produces a more severe somatic phenotype.

Analysis of the haematological data in the Irish traveller group suggests that there are 4 distinct groups in this population:

1. Fanconi anaemia mosaic and absolute absence of Natural Killer cells.
   They are at risk of developing HLH but bone marrow failure either does not occur or is a very late development

2. Fanconi Anaemia mosaic (with reduction in NK numbers)

Bone marrow failure occurs but the age of occurrence is variable and may be later than the rest of the FA group

3. Fanconi Anaemia

   Bone marrow failure occurs between 40-65 months of age and transfusion dependence between 60 – 70 months of age. There is a risk of early development of AML/MDS. The risk seems to relate to particularly early development of bone marrow failure (before 40 months).

International data suggests that the mean age of onset of haematological abnormalities is 7.6 years although children with null mutations of the FACA gene will have earlier onset. (Faivre et al, 2000) In our group (excluding the group 1 FA mosaics who appear to behave differently) it appears that haematological abnormalities develop much earlier at a median of 52 months and have progressed to transfusion dependence by 60-70 months. Platelet dependence seems to occur initially followed within 6 months by red cell dependence. In the EUFAR study only 25% of FACA null mutations will have developed haematological abnormalities by 60 months (Faivre et al, 2000). Data extrapolated from recent International Fanconi Anaemia Registry paper suggests that although 80% of individuals with FA complementation group A will have developed bone marrow failure by 120 months, only 35% have bone marrow failure by 60 months (Kutler et al, 2003b).

One boy (child 11) whose older sister had also died of FA (no data available) had a particularly aggressive course, developing haematological abnormalities at 20 months and transfusion dependence at 36 months. His first cousin (child 2) also had an earlier onset of bone marrow failure than is usual for this group. Child 11 developed AML by 95 months and as child 2 was displaying a more aggressive course he may also been at risk of developing early leukaemia, however he had undergone BMT by 89 months. It is unclear what separates these 2 children from the rest of the FA group. They may have other gene abnormalities but we know that they must have had FA-A del 11-14 because child 3, a sibling of child 2, has this gene defect.

Two other children transplanted at 108 and 114 months had no evidence of clonal evolution at the time of transplant. Haematological malignancy is the commonest cancer

in FA and has a median age of onset of 11.3 years (Rosenberg et al, 2003). Our data suggests that AML/MDS could occur in our group significantly earlier than predicted by European or international data. It has been suggested by one group evaluating poor risk groups of FAC populations that they should have yearly bone marrow aspirations for morphological examination and cytogenetics analysis prior to the onset of haematological abnormalities and thereafter 6 monthly examination because of the higher risk of leukaemia development (Gillio et al, 1997). Our group have a similar aggressive course would benefit from this approach.

One of the children with FA mosaicism developed an EBV related lymphoproliferative disease at 35 months. This type of disease has only been documented in one individual with FA who was receiving cyclosporin post-BMT and suggests that it may be related to his concomitant Natural Killer cell deficiency (*cf* Chapter 5) (Rosenberg et al, 2003).

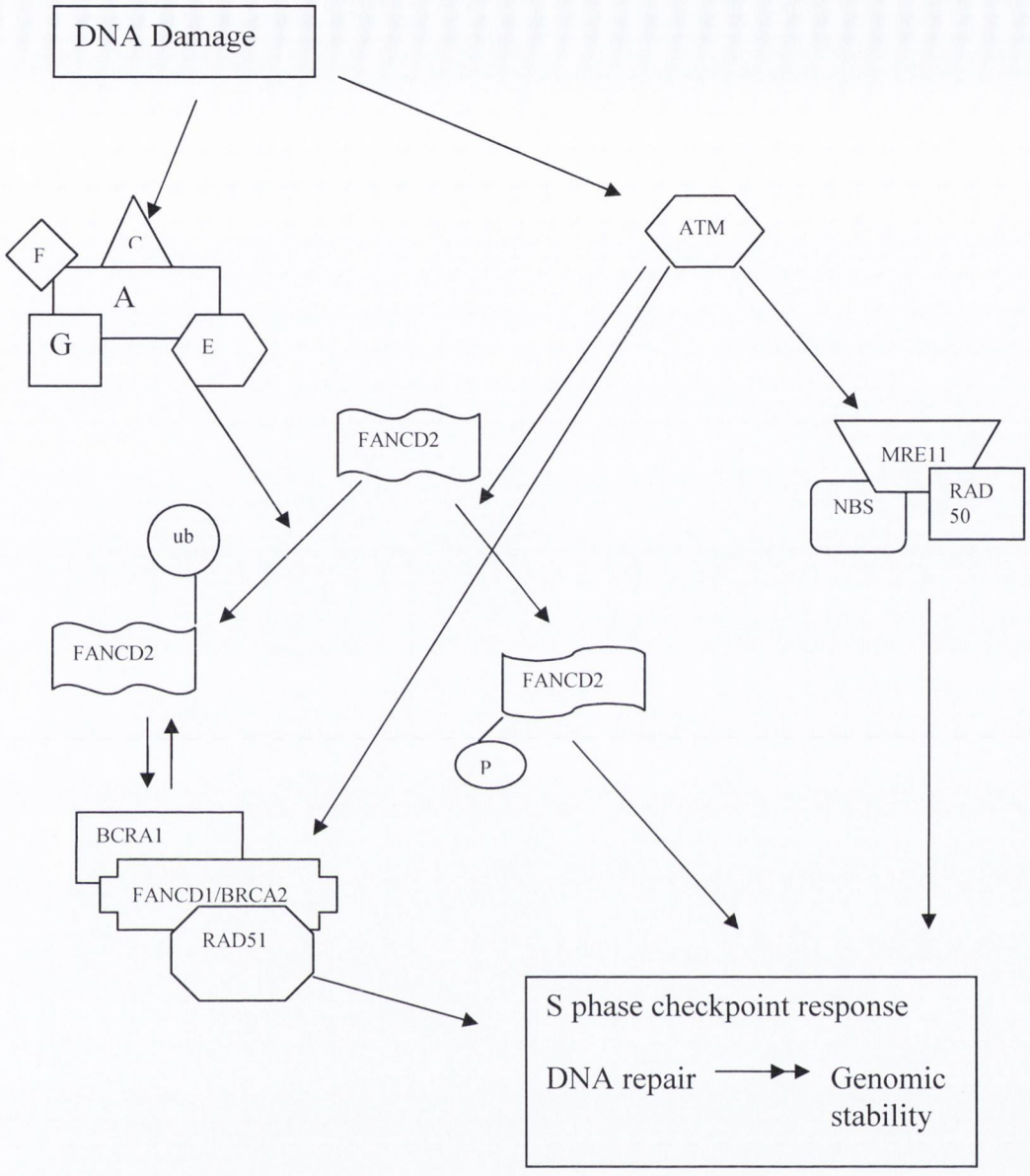
The conditioning regimen used in this group is intermediate dose cyclophosphamide (Cy) without irradiation. This was first shown to be effective by the Seattle group (Flowers et al, 1996). Many other groups continue to use lower dose cyclophosphamide (20mg/kg total dose) and irradiation but it is suggested that cancer development post-transplant (which has an 8 year projected incidence of 24%) may be related to irradiation and some groups are suggesting that it should not be used (Socie et al, 1998). Currently all the children in our group are alive at a median of 43 months (11-72 months) post transplant but are carefully monitored for the development of cancer particularly squamous cell carcinoma of the upper airways to which they are particularly susceptible (Kutler et al, 2003a). The total dose of cyclophosphamide (Cy) used in our group was 120mg/kg. This has not produced significant toxicity although one child developed haemorrhagic cystitis which may be related to altered cyclophosphamide metabolism and reduced clearance (Yule et al, 1999). Only one child developed significant GVHD which required treatment with high dose steroid. The Italian group who analysed data on 27 patients, 5 of whom received Cy at a dose of 120mg/kg found that this dose was associated with an increased rate of acute GVHD (aGVHD) (Dufour et al, 2001). However more recently the findings of another European group who use thoraco-abdominal irradiation and low dose cyclophosphamide (20mg/kg total dose) have cast doubt on this as they found analysing the likelihood of aGVHD in children transplanted for acquired aplastic anaemia or FA,

that FA itself was a significant predictor for the development of aGVHD and that it was probable that 89% of FA patients less than 12 years of age at the time of transplant would receive steroids by day +100 post-transplant. They did not find that time to transplant, number of pre-transplant transfusions or androgen usage were significant predictors of aGVHD. They suggested that donor/recipient major ABO blood group incompatibility and urogenital malformations increased the likelihood of corticosteroid resistant aGVHD (Guardiola et al, 2003). This group previously reported that in addition to chronic GVHD, more than 20 pre-transplant transfusions had an adverse impact on survival, suggesting that transfusion dependence should be a trigger for bone marrow transplantation (Socie G et al, 1998).

Conclusions

Fanconi anaemia in the Irish traveller population is caused by a large gene deletion in the FANCA gene producing a null gene which does not produce protein. It is associated with more somatic mutations and earlier bone marrow failure than other null mutations in the FANCA gene. In at least one child FA had an aggressive course, culminating in the development of early MDS/AML and death. It is imperative that this diagnosis is considered in children from these kindreds who present with dysmorphism, abnormal skin pigmentation or failure to thrive. Children in whom FA is suspected should have early referral to a paediatric haematology centre, where definitive diagnosis, including Fanconi genotyping, can be made. Those with FA should be tissue typed and donors identified. They should have regular, possibly 6 monthly, haematological assessment to determine the onset of haematological abnormalities and there after more frequent monitoring. Regular bone marrow examination, as previously described, would allow early detection of clonal evolution and contribute to the determination of the correct timing of BMT. Bone marrow transplantation should be undertaken as soon as possible following the onset of bone marrow failure. Careful long-term follow-up of those who have been transplanted with particular attention to the development of head and neck oesophageal and gynaecological solid tumours is imperative as it appears that they may be more common following transplant.

Fig 4.1



A schematic diagram showing FA protein complex and central role of FANCD2 in DNA damage response pathway.

DNA damage caused by Crosslinking agents such as MMC activates FA protein complex leading to monoubiquitination of FANCD2, which interacts with BRCA1 to effect DNA repair. Damage caused by ionising radiation activates ATM which phosphorylates FANCD2 in addition to several other proteins to coordinate the S phase checkpoint response. (From Tischkowitz MD Hodgson SV. J Med Genet 2003;40:1-10)

Table 4.1 Patient demographics

Patient No.	Sex	Age at presentation to Tertiary centre (months)	Reason for Diagnosis	Method of diagnosis	Hb at presentation (g/dl)	WCC at presentation (x10^9/l)	Neutrophil count at presentation (x10^9/l)	Platelet count at presentation (x10^9/l)
1	M	65	SS/FTT	DEB	9.3	2.7	1.08	34
2	M	41	SS/Speech delay	DEB	10.9	5.8	n/a	42
3	M	2	FH/Appearance	DEB	12.5	7.7	2.1	235
4	F	59	Bruising	DEB	9.7	3.9	1.09	39
5*	F	59	FH	DEB	11.2	6.4	2.07	146
6	M	2	FH/Thrombocytopenia	DEB	11.6	N/A	1.8	286
7	F	1	FH/appearance	DEB	16.1	11.1	2.6	344
8	F	44	SS	DEB	11.0	14.6	5.9	159
9*	F	52	Thrombocytopenia	DEB	8.9	2.9	0.6	30
10	F	12	FH/FTT	Fibroblasts	11.6	9.2	1.7	318
11	M	20	FTT/Bruising	DEB	11.3	10.6	N/A	135
12*	M	21	HLH	DEB	9.7^{+}	8.0	N/A	120
13*	F	84	FH	DEB	11.6	15.3	6.58	624
14*	M	36	HLH	DEB	8.3	2.79	1.9	154

Key: No., number; Hb, haemoglobin; g/dl, grams per decilitre; *, Fanconi Mosaic; SS, short stature; FTT, failure to thrive; FH, family history; HLH, haemophagocytic lymphohistiocytosis; DEB, diepoxybutane stress test; N/A, not available; +, post transfusion.

Table 4.2 Physical features of the study group

Patient No.	Short stature	Abnormal pigmentation	Microcephaly	Typical facies	Thumb/hand anomalies	Ear anomalies	Cardiac abnormalities	Abnormal Genitalia	Renal Anomalies
1	Y	Y	Y	Y	Y	N	N	N	N
2	Y	Y	Y	Y	Y	Y	N	Y	Y
3	Y	Y	Y	Y	Y	Y	N	N	Y
4	N	Y	N	N	N	N	N	N	N
5*	N	Y	N	N	Y	N	N	N	N
6	Y	Y	Y	Y	Y	Y	Y	Y	Y
7	Y	Y	Y	Y	Y	Y	Y	N	N
8	Y	Y	Y	Y	Y	Y	Y	N	N
9*	N	Y	Y	Y	Y	N	Y	N	N
10	Y	Y	N	N	Y	N	N	N	N
11	Y	Y	Y	Y	Y	N	N	N	Y
12*	Y	Y	Y	Y	Y	N	N	N	N
13*	Y	Y	Y	Y	N	N	N	N	N
14*	N	Y	N	N	N	N	N	N	N
Total %	73%	100%	73%	73%	80%	33%	26%	13%	26%

Key. No., number; Y, yes, N, no; * Fanconi Mosaic; %, percent

Table 4.3 Complications and treatment

Patient No.	Age at most recent evaluation	WCC <1.0x10^9/l	Hb<10g/dl	Platelets <100x10^9/l	Age at 1st RCC Transfusion	Age at 1st Platelet transfusion	Age commencing Androgens	Age at BMT	Age developing AML/MDS	Age developing 2° cancer	Age at death
1	177mo	54mo	54mo	43mo	84mo	75mo	89mo	108mo	-	-	-
2	174mo	DK	46mo	36mo	55mo	44mo	43mo	89mo	-	-	-
3	128mo	50mo	64mo	36mo	66mo	-	64mo	73mo	-	-	-
4	153mo	83mo	59mo	?	66mo	62mo	72mo	83mo	-	-	-
5	128mo	96mo	96mo	35mo	104mo	112mo	-	114mo	-	-	-
6	21mo	-	-	-	-	-	-	-	-	-	-
7	8mo	-	-	-	-	-	-	-	-	-	-
8	97mo	91mo	82mo	48mo	93mo	96	-	97	-	-	-
9	96mo	By 52mo	By 52mo	By 52mo	60mo	58mo	-	78mo	-	-	-
10	34mo	-			-	-	-	-	-	-	-
11	n/a	52mo	37mo	29mo	38mo	36mo	72mo	-	95mo	-	99mo
12	85mo	-	20mo#	20mo#	20mo#	-	-	-	-	35mo	-
13	141mo	-	-	-	-	-	-	-	-	-	-
14	38*mo	NR	NR	NR	NR	NR	-	-	-	-	38mo

Key: No., number; RCC, red cell concentrate; BMT, bone marrow transplantation; AML, acute myeloid leukaemia; MDS, myelodysplastic syndrome; 2°, secondary; mo, months; -, did not occur; #, transient, related to acute illness; *, age at death; NR, not relevant as not related to FA.

Table 4.4 Transplantation details

Patient No.	Sex	Age at transplant (months)	Type of transplant	Conditioning regimen	GVHD Prophylaxis	Problems during transplant	Outcome	Time elapsed since transplant (months)
1	M	108	FD	Cyclophosphamide 30mg/kg x 4days	Cyclosporin MTX	N	Well	60
2	M	89	Sib	Ditto	Ditto	N	Well	72
3	M	73	Sib	ditto	Ditto	Haemorrhagic cystitis	Well	36
4	F	83	FD	Ditto	Ditto	VOD	Well	60
5	F	114	FD	Ditto	Ditto	GVHD	Well	10
9	F	78	FD	Ditto	Ditto	N	Well	16

Key; No., number; GVHD, graft versus host disease; M, male; F, female; FD, family donor; Sib, sibling; mg/kg, milligrams per kilogram; MTX, methotrexate; N, no; VOD, veno-occlusive disease

References Chapter 4

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Chapter 5

Haemophagocytic Lymphohistiocytosis and Fanconi Mosaicism

Natural Killer cells.

Natural killer (NK) cells are a distinct subset of lymphocytes, morphologically resembling large granular lymphocytes, that have the capacity to mediate spontaneous lysis of sensitive target cells including certain tumour cells, virus infected cells and haematopoietic cells. They do this in a non-MHC restricted fashion and mediate antibody-dependant cellular cytotoxicity (ADCC), but without killing normal cells. They comprise 10-15% of normal peripheral blood mononuclear cells and are derived from a common lymphoid precursor cell (Robertson et al, 1990). They may be derived directly from precursors in the bone marrow and may transit through the thymus although the thymus is not required for their development. IL15 is critical for the development of mature NK cells from NK progenitors in the bone marrow and for maintenance of NK cells in the periphery (Heusel et al, 2003). Phenotypic and functional NK cells may arise from immature CD7+,CD1-,TcR-,CD3-,CD4-,CD8-, prothymocytic precursors and express CD2. CD2 is thought to be important in NK cell adhesion and activation. Most NK cells also express CD16 (a low affinity receptor for the Fc region of IgG) which although not directly involved in cell killing mediates ADCC. CD56 antigen is used most often as an NK marker. It is expressed by almost all human peripheral blood cells capable of non-MHC-restricted cytotoxicity. It appears not to participate directly in NK killing of target cells although some reports suggest that it can mediate homotypic adhesion of NK cells to CD56+tumour cell lines. NK cells are best defined phenotypically as CD56+CD3- lymphocytes (Robertson, 2002). (Table 5.1).

NK-mediated cytotoxicity does not require prior sensitisation or antigen presentation for target cell lysis and so provides early host defence from infection. NK mediated lysis is enhanced by IL2, IL12, TNF and IFNγ. (fig 5.1) NK cells are able to lyse cells deficient in MHC expression unlike cytotoxic T lymphocytes (CTLs). NK recognition and activation appear to be dictated by a balance between inhibitory and activating receptors

on the NK surface. (*cf* chapter 8). Inhibitory receptors, represented by p58 and p70 receptors of the immunoglobulin superfamily, recognise autologous MHC class1 molecules expressed on normal cells or other NK-cell-resistant targets (Bierer et al, 1996). Binding of these inhibitors to their ligands causes recruitment and activation of intracellular phosphatases (e.g. SHP-1), leading to dephosphorylation of molecules that normally signal activation (Wallin et al, 2003). Down regulation of MHC class I molecules on the target cell surface which commonly occurs during viral infection or neoplastic transformation, releases the NK cell from inhibitory signals and allows lysis of the aberrant target cell (Robertson, 2002).

NK cells induce cell death by 2 different mechanisms;

(1) MHC independent pathways using a perforin complex on the surface of the NK cell to disrupt the cell membrane. Resting NK cells have preformed cytolytic granules that can be discharged immediately after exposure to sensitive target cells. Granule exocytosis releases perforins, serine esterases, granzymes and chondroitin sulphate proteoglycans into areas bounded by the close apposition of effector cell and target cell plasma membranes. Perforins (also called pore forming proteins) are inserted into the target cell membrane as monomers and subsequently polymerise to form cylindrical transmembrane pores that either permits osmotic lysis of the target cell (Robertson, 2002), or allows granzymes to enter the cell and trigger an "internal disintegration" pathway leading to target cell damage (fig 5.2). Granzyme B has been shown to disregulate the normal cell cycle processes by activating $p34^{cdc2}$ kinase which initiates the dissolution of the nuclear envelope to produce a "mitototic catastrophe" that may account for apoptotic nuclear damage (Henkart, 1994).

(2) Fas ligand (FasL) and tumour necrosis factor related apoptosis inducing ligand (TRAIL) induces cytotoxicity possibly by upregulating Fas and activating a target cell endonuclease that degrades genomic DNA, promoting apoptosis (Wallin et al, 2003; Robertson, 2002). These actions are prevented by inhibitory signals mediated by MHC class 1 recognition. Crosslinking of the activation receptor NK1.1 on NK cells leads to upregulation of FasL on the cell surface but simultaneous engagement of an MHC class

1 binding inhibitory receptor prevents externalisation of FasL (Wallin et al, 2003). It is suggested that the Fas pathway is principally involved in regulating immune responses (Henkart, 1994).

In addition to a role in immune surveillance, NK cells protect from viral infections, which is particularly important in the early phases of the immune response before the recruitment of T-cells. The expression of viral antigens or other surface structures by infected cells appears to render them more sensitive to NK cytolysis. Decreases in NK cells have been documented in a number of disorders (Table 5.2) including Chediak-Higashi syndrome, leukocyte adhesion (CD18) deficiency and X-linked lymphoproliferative syndrome (Bierer, 1998). It has been shown that NK cells can inhibit malignant tumour growth in vivo under experimental conditions and although it is difficult to demonstrate that NK cells are involved in immune surveillance against spontaneously arising autologous tumours in man, there is an increased carcinogenic risk in those with NK deficiency. Some of this may reflect inadequate NK antiviral activity with subsequent EBV-induced lymphoproliferation but an association of NK with other malignant tumours has also been documented (Robertson, 2002).

Complete deficiency of NK cells has been associated with severe recurrent infections with herpes viruses (varicella, simplex, CMV) and recurrent otitis media (Biron et al, 1989).

Fanconi Anaemia Mosaicism

Hypersensitivity to DNA cross-linking agents such as DEB and MMC in mitogen stimulated peripheral blood lymphocyte cultures is an essential diagnostic criterion of Fanconi Anaemia (FA). Individuals heterozygous for the FA gene have normal clastogenic stress tests. (Rosendorff et al, 1988) However in 10-20% of FA patients there is a mixed population of lymphocytes with hypersensitivity and normal sensitivity to DNA cross-linkers. This finding suggests that a reversion to wild-type phenotype spontaneously occurs in a subpopulation of lymphocytes. Analyses of mosaicism in compound heterozygotes for pathogenic FANCC mutations indicate that intragenic mitotic recombination or gene conversion restores a wild-type allele. Study of a group

of patients with FANCA due to microdeletion, microinsertion and missense mutations demonstrated functional correction by compensatory mutations in cis, whilst in a second group of FANCA patients, with compound mutations in the FANCA gene, one of 2 constitutional point mutations reverted to the original wild type sequence (Yamashita et al, 2001). The majority of the reversions took place in the region of exons 10 to 11 which is known as a highly mutable region due to an abundance of repetitive elements. This self correction confers a proliferative advantage and the progeny of the self-corrected precursor cell can be expected to slowly replace the defective cell population (Gross et al, 2002).

Fanconi Anemia mosaicism has clinical implications. The results of DEB or MMC using peripheral blood lymphocytes can be difficult to interpret and fibroblasts should be used for diagnostic tests. Mosaicism in lymphocytes may be associated with mosaicism in haematopoietic stem cells which may prevent the onset and/or progression of bone marrow failure. However, the attenuated conditioning regimens for bone marrow transplantation may fail to eliminate reverted cells, leading to graft rejection in FA mosaic patients (Yamashita at al 2001).

Reduced NK cells have been reported in individuals who are homozygous and heterozygous for FA (Froom et al, 1987; Petridou et al, 1990; Heim et al, 1992). It is suggested that this defect may lead to increased susceptibility to viral infection and contribute to the development of malignant disease in these conditions (Petridou et al, 1990).

Haemophagocytic lymphohistiocytosis

Haemophagocytic lymphohistiocytosis (HLH) is a reactive disorder of the mononuclear phagocyte system and is defined by a constellation of clinical and haematological abnormalities, usually appearing in infants and young children (table 5.3). The patients are acutely ill with high fever, hepatosplenomegaly, lymphadenopathy, cytopenia and bone marrow haemophagocytosis without signs of malignancy. Laboratory features include hypofibrinogenaemia, hyperferritinaemia, and hypertriglyceridaemia (Henter et al, 1991). The characteristic histopathological finding is diffuse infiltration primarily of the liver, spleen, lymph nodes and brain by activated non-Langerhans histiocytes

actively phagocytising blood cells (Egeler et al, 1996). .Two distinct forms have been recognised: a familial form which is inherited as an autosomal recessive disorder, in which at least 80% of cases present before the age of 2 years and a secondary form which occurs in association with systemic infection, underlying malignancy or an immunodeficiency syndrome (Hirst et al, 1994). Most patients with HLH have impaired natural killer cell (NK) activity, suggesting that the disease arises from a selective immune deficiency that compromises the body's defence mechanisms against infections (Egeler et al, 1996). There is T-cell activation and clonal expansion but the activated cells fail to kill the infected cells and remove the source of antigen stimulation. Persistent antigen driven T cell activation results in the production of large quantities of cytokines, including interferon γ (IFN-γ) and granulocyte-macrophage colony-stimulating factor (GM-CSF) both of which are known macrophage activators. The sustained activation of macrophages and T cells results in tissue infiltration and the production of high levels of tumour necrosis factor α (TNF-α), interleukin 1 (IL-1) and IL6, cytokines that play a major role in the tissue damage and clinical symptoms characteristic of HLH (Arico et al, 2002). In the absence of another diagnostic nonclinical parameter, NK and cytotoxic T lymphocyte function are reliable measures to support the diagnosis of HLH (Schneider et al, 2003).

For many years a specific genetic marker of HLH could not be identified until the demonstration of a link between the disease and chromosome region 9q21.3-22 and a more common second locus in the 10q21-22 region. Stepp et al identified mutations in the perforin gene (PRF1) in eight unrelated patients (Stepp et al, 1999). More recently further mutations in PRF1 have been identified in patients of different ethnic origin (Graham et al, 2000; Clementi et al, 2001; Goransdotter Ericson et al, 2001). This suggests that PRF1 mutations serve as a definitive marker of familial HLH and are thought to produce their effect by compromising the cytolytic activity of killer lymphocytes by interfering with the co-ordinated action of perforin and granzymes (Arico et al, 2002). The differential diagnosis includes X-linked lymphoproliferative disease which develops in boys with a mutation in the SH2D1A gene, following infection by Epstein-Barr virus. These children have normal perforin expression but NK function is impaired due to the presence of inhibitory 2B4 receptor as a result of the altered SAP gene (Arico et al, 2002). Arico et al have proposed a diagnostic algorithm which would help distinguish familial HLH from viral associated lymphohistiocytosis

and X-linked lymphoproliferation based on perforin expression by peripheral lymphocytes, NK activity and 2B4 lymphocyte receptor (Fig 4.3). It is further suggested that assessment of NK function and perforin expression in other family members may be potentially useful in early diagnosis prior to onset of the haemophagocytic process in FHL (Egeler et al, 1996; Kogawa et al, 2002).

Management strategies for Children with HLH

Early bone marrow transplantation is the treatment of choice for individuals with familial HLH. In the absence of a fully matched family donor, matched unrelated donor transplantation has proved effective. Active disease or incomplete resolution of disease prior to transplant has not been shown to impede successful outcome (Durken et al, 1999). Management strategies for those with sporadic HLH involve immune modulation. Therapies which have been employed include intravenous immunoglobulin (ivIg) (Goulder et al, 1990), pulses of high dose steroid, plasma exchange (Kodama et al, 2000), cyclosporin, anti-thymocyte globulin (ATG) and Etoposide (Chen et al, 1995; Hirsh et al, 1994).

The overall survival at 5 years, in 2 large longterm studies, for sporadic HLH was 36% with aggressive management and familial HLH was uniformly fatal without bone marrow transplantation (Hirst et al, 1994; Chen et al, 1998). The main causes of death were overwhelming bacterial or viral infection and/or haemorrhage. An age of less than 2 years at presentation was a significant predictor of mortality (Hirst et al, 1994). In Chen's group 3 patients progressed to T-cell lymphoma, characterised in 2 cases by clonal chromosomal abnormalities. Epstein-Barr virus infection was implicated in the disease pathogenesis (Chen et al 1998).

Neurological abnormalities including irritability, depressed level of consciousness, hypotonia and convulsions were noted in 30% of patients in Hirst's group from Kings and Great Ormond Street Hospitals. However cerebrospinal fluid (CSF) abnormalities, which included pleocytosis with mononuclear cells some showing evidence of haemophagocytosis, were seen in 76% of patients. One to two courses of intrathecal methotrexate produced complete resolution of the CSF findings and neurological abnormalities (Hirst et al, 1994).

Cases, Materials and methods.

Between 1997 and 2003, 3 children from different Traveller families were referred to the Haematology departments of the National Children's Hospital and Our Lady's Hospital for Sick Children, Dublin for investigation and management of lymphadenopathy and hepatomegaly. The families knew of no specific family relationship however all 3 families were from the same interrelated family groups. Histological examination of lymph nodes, bone marrow and liver demonstrated haemophagocytic lymphohistiocytosis. All of the children had NK cells analysed, one child had perforin levels measured and 2 of the 3 had clastogenic stress testing for FA.

Methodology for Natural Killer Cell Analysis.

Peripheral blood is collected in EDTA (Etylenediamine-tetraacetic acid) and stored at room temperature for less than 24 hours. A white blood cell (WBC) count and differential white cell count is obtained. Twenty microlitres of BD MultiTEST CD3/4/8/45 reagent and 20mcl BD MultiTEST CD3/56+16/45/19 are placed in Trucount tubes. Fifty microlitres of blood or control is added and the mixture is vortexed and incubated for 15 minutes in the dark. Four hundred and fifty microlitres of FACSLysing solution is then added to each tube, vortexed and again incubated for a further 15 minutes in the dark. The samples are then analysed on a FacsCalibur flow cytometer (Becton Dickinson). The absolute count is determined by:

$$\frac{\text{No. of events in the region containing the cell population}}{\text{No. of events in absolute count bead region}} \times \frac{\text{No. beads per test}}{\text{test volume of cell population}} = \text{Abs Count}$$

Methodology for measurement of peripheral blood mononuclear cell Perforin expression.

Peripheral blood mononuclear cells (PBMC's) were prepared by density gradient separation using Lymphoprep™ (Axis-Shield). One hundred microlitres of 1 X 10^6/ml of these cells were surface stained with CD45 APC. They were incubated for 15mins and then washed in Cell Wash™. The cell surface was fixed using the Fix&Perm® kit (Caltag Laboratories) and the membrane permeabilised. Twenty microlitres of anti-human Perforin (Becton Dickinson) was added to the cell suspension and 20µl isotype negative control was added to a control tube. Both tubes were incubated for 20mins, and then washed twice in CellWash ™. The cells were suspended in fixative (containing 1% paraformaldehyde) and analysed immediately on a FacsCalibur flow cytometer (Becton Dickinson).

Case Histories:

Case 1

Child A, a male child, was referred to the haematology department aged 5 years for evaluation of ongoing failure to thrive (Height and weight less than 3rd percentile), hepatosplenomegaly, lymphadenopathy, anaemia (7.9g/dl), neutropenia (1.0g/dl) and a non-specific lymph node histiocytosis.

He had a past history of neonatal sepsis, and initially presented at 1 year with failure to thrive, fluctuating hepatosplenomegaly, lymphadenopathy and congestive cardiomyopathy. He had multiple hospital admissions with respiratory tract infections. He had been noted to have an absolute NK cell deficiency. He was the child of a consanguineous marriage and had one younger brother, who was well.

Clinical evaluation demonstrated short stature and microcephaly but no skeletal abnormalities. The clinical findings of hepatosplenomegaly, lymphadenopathy, the laboratory demonstration of pancytopenia and hypertriglyceridaemia (3.09 {.23-1.7}) and the tissue histiocytosis supported a diagnosis of HLH. Examination of cerebrospinal

fluid failed to demonstrate any abnormalities. He was commenced on Prednisolone 1mg/kg/day and Cyclosporin A 5mg/kg/day.

The hepatosplenomegaly, lymphadenopathy and pancytopenia resolved over the following 6 months but he continued to have recurrent herpes simplex and non-specific upper respiratory tract infections. Initially the prednisolone was weaned and discontinued without disease relapse but the cyclosporin therapy was then discontinued and within one month his pyrexia, lymphadenopathy, and hepatosplenomegaly had recrudesced. The cyclosporin was recommenced and the organomegaly and lymphadenopathy again resolved within 1 month. Repeat bone marrow aspirate and biopsy performed at this time was normal. Nevertheless he continued to have monthly viral infections and also developed 2 episodes of chicken pox. Allogeneic bone marrow transplantation was discussed but rejected as his overall clinical condition was good. Two years later he developed life threatening interstitial pneumonitis, hepatic dysfunction and an addisonian crisis. Recovery from this was followed in quick succession by 3 further herpes simplex infections and an emergency admission with gastroenteritis.

He underwent allogeneic bone marrow transplantation from his histocompatible brother 6 months later, aged 8 years, at the Regional Centre for Immunological Diseases, Newcastle. The conditioning regimen used was Busulphan (16mg/kg/day x 4 days), Cyclophosphamide 100mg/kg/day x 4 days and Antithymocyte Globulin (ATG) (5mg/kg/day x 5 days). Acyclovir (10mg/kg, three times a day intravenously) and Co-trimoxazole (240mg, twice a day by mouth after neutrophil engraftment of $>0.5x10^9/l$) were given as prophylaxis against Herpes virus infections and Pneumocystis carinii infections, respectively. Cyclosporin A was given intravenously then orally at a dose producing a 12 hour trough plasma level of between 100 and 200 mcg/l for 1 year post-BMT as graft versus host disease (GVHD) prophylaxis.

The year following transplant was complicated by recurrent respiratory and herpes simplex infections, however he has had no hospital admissions in the past 3 years and remains very well. DNA analysis confirms full donor chimera and NK cells are now normal. He remains on the 3rd centile for height and weight and has obvious microcephaly. Fibroblast culture to exclude the possibility of a predisposing Fanconi

Anaemia is now planned in view of our new knowledge of the disease expression in this group of children.

Case 2

Child B was referred at 20 months of age with pyrexia, hepatosplenomegaly, lymphadenopathy and pancytopenia. He had been born by elective Caesarean section at 34 weeks gestation because of intrauterine growth retardation and initially investigated at 9 months for failure to thrive. His mother and father were cousins. His oldest sibling had died, aged 2 years, of a possible adrenal crisis and overwhelming sepsis. She had also been investigated for failure to thrive. His surviving sister (Child D) was believed to have adrenal insufficiency and had been on steroid therapy since shortly after birth. She was dysmorphic and also had failure to thrive. Child B was less than the 3rd centile for height and weight. He had microcephaly and micropthalmia but no radial ray defects or renal abnormalities. He was markedly hyperpigmented.

His Haemoglobin (Hb) on admission was 5.1g/dl and platelets were 15x10^9/l. His immunoglobulin levels were normal but NK cells were not detectable. Triglycerides were elevated but fibrinogen was normal. Epstein Barr titres showed IgM positivity. Bone marrow aspirate, biopsy, lymph node and liver biopsy demonstrated an infiltrate of histiocytes consistent with the diagnosis of HLH. He was commenced on Prednisolone and made a prompt recovery. The steroid therapy was discontinued within 3 months of diagnosis. Repeat NK cell measurement failed to demonstrate any improvement.

A Fanconi screen, done because of his microcephaly and failure to thrive, showed Fanconi mosaicism. His sister (Child D) also had Fanconi mosaicism and reduced (25% of normal) levels of NK cells. She has had multiple admissions with respiratory tract infections but has never developed HLH.

One year following initial presentation, child B was admitted with sudden onset of vomiting and abdominal pain. He was noted to have abdominal distension and a plain x-ray of abdomen demonstrated air:fluid levels. He was treated conservatively with a nasogastric tube and intravenous fluids and the obstruction resolved. An abdominal

ultrasound failed to demonstrate any abnormality. The following month he again presented with diarrhoea and vomiting. Repeat abdominal x-rays were normal and the symptoms again resolved. He had 2 further admissions over a 2 month period with vomiting and abdominal distension. On the last occasion he failed to settle with conservative measures and a barium enema was performed. This demonstrated a high grade small bowel obstruction but no masses were seen. He then underwent a laparotomy, was noted to have a stricture and had an ileal resection performed. Histological examination of the resected area demonstrated mucosal ulceration associated with a dense infiltration of large atypical cells involving the submucosa, muscularis mucosa and lamina propria. These cells expressed both B and T cell markers and immunohistochemistry demonstrated the presence of Epstein Barr virus in many of the cells (Fig 5.4, 5.5, 5.6, 5.7, 5.8). A diagnosis of an EBV-associated lymphoproliferative disorder, of a type frequently associated with immunodeficiency was made. Tumour staging failed to demonstrate any extension beyond the margins of the resected area. Child B had no histocompatible sibling donor and unrelated donor searching was also unsuccessful, he was, therefore, not a suitable candidate for bone marrow transplantation. He has received no specific therapy for his lymphoproliferative lesion; nevertheless he has been well for the past 5 years without recurrence of either the HLH or the lymphoproliferation. Plans to analyse perforin expression and PRF1 mutations have had to be deferred because of recent clinic non-attendance.

Case 3

Child C, aged 3 years, was admitted with a 3 week history of pyrexia, anorexia and vomiting. He had initially been admitted to another hospital with anorexia and pyrexia. A diagnosis of "viral infection" had been made but he had failed to improve.

His mother and father were 2nd cousins and his social circumstances were poor. There was an extended family history of hepatic tumours, cardiomyopathy and leukaemia. Child C was born at 37 weeks gestation by elective caesarean section as he was a breech presentation. He had been hospitalised on 3 occasions with viral infections. His first admission had been at 6 weeks of age.

The clinical examination on admission revealed him to be pyrexial and pale. He had diffuse lymphadenopathy and hepatosplenomegaly. He had a systolic murmur all over the praecordium and coarse crackles throughout both lung fields. Laboratory results showed an anemia, neutropenia and hypofibrinogenaemia. His liver function was abnormal (Table 5.4).

His condition continued to deteriorate over the following 6 days. He developed cardiac failure and his ejection fraction was 21%. A potential Fanconi Anaemia familial relationship was noted and he had T, B and NK cells measured (table 5.5). A bone marrow aspirate and biopsy and lymph node biopsy were performed (fig 5.9, 5.10), all of which demonstrated extensive haemophagocytosis. He was noted to have hyperferritinaemia (4939 {7-150}) and hypertriglyceridaemia (3.61 {0.23-1.7}). Epstein Barr antibody results showed IgM positivity and EBV DNA was isolated (10,000 copies/ml). He had perforin studies performed which showed a lack of perforin expression (fig 5.11). A diagnosis of haemophagocytic lymphohistiocytosis was made and he was commenced on Acyclovir 500mg/m^2 tds, ivIg 500mg/kg, methylprednisolone 2mg/kg/day and cyclosporin A 2.5mg/kg/day. Fresh frozen plasma was administered to correct the coagulopathy.

He showed some improvement over the next 12 days. His haematological parameters normalised and although the hepatosplenomegaly persisted it too had reduced in size. He then developed acute pulmonary oedema and renal failure requiring ventilation and haemofiltration. Cyclosporin A and ivIg were stopped and methylprednisolone was reduced to 1mg/kg/day and then halved every 2 days until he received a dose of 0.25mg/kg/day. He initially improved again but within 8 days he had developed abnormal jerky movements necessitating CT scanning and cerebrospinal fluid examination; both of which were normal. His pyrexia recurred and by day 22 of treatment, he had an extensive left sided pulmonary consolidation. The hepatosplenomegaly persisted. An open lung biopsy was performed which showed extensive histiocytosis. Cyclosporin A and ivIg were recommenced and methylprednisolone was increased again but he continued to deteriorate with worsening pulmonary consolidation and effusion, enlarging lymphadenopathy and hepatosplenomegaly. Rabbit Antithymocyte globulin (10mg/kg/day x 5days) and Rituximab (375mg/m^2 once/week) were added on day 28. Cyclosporin A was

increased to 5mg/kg/day and methylprednisolone increased to 3mg/kg/day. This failed to halt the deterioration and he had a cardiorespiratory arrest on day 30. A post mortem examination was refused. The results of lymphocyte clastogenic stress testing which became available shortly after child C's death demonstrated elevated levels of chromosome fragility in the unstressed and DEB treated cultures but there was no gross hypersensitivity to DEB, unlike that normally found in Fanconi Anaemia.

Investigation of both his parents and 2 siblings revealed that both parents have reduced absolute NK cell numbers to half normal values (217 and 156 x10^9/l {300-700}) and one female sibling has one third normal values (80x10^9/l). All of the family have normal clastogenic stress testing.

Discussion

Haemophagocytic lymphohistiocytosis is a rare clinical condition that can arise as a viral driven phenomenon (Chen et al, 1995), or as a familial form produced by perforin deficiency and related to mutations in the PRF1 gene (Stepp et al, 1999). The familial form has been diagnosed in the past based on a history of consanguinity or a previously affected sibling. In eighty percent of cases it occurs in children less than 2 years of age and is most frequently fatal (Hirst et al, 1994). This group of children are all from consanguineous relationships and although the families are not obviously related they are families who are known to have intermarried in the past and in whom there is also a history of Fanconi anaemia. This suggests that these children may have familial HLH (FLH). Child A and B presented at 12 and 20 months respectively whilst child C, who succumbed to the disease, presented at 3 years. All had a classical presentation but diagnosis of the condition was delayed in all 3 cases. Child B and C had a history of EBV infection which most likely precipitated their disease, however child A never had these titres checked during his acute presentation and its role in precipitating his disease is unknown.

All 3 children had an absolute deficiency of NK cells which probably predisposed them to FLH (Schneider et al, 2003). It is known that absolute NK deficiency predisposes to severe recurrent infection by the herpes virus group (Biron et al, 1989), and it is possible that this "double hit" produced the HLH syndrome in our patients. Interestingly

child B's sibling (Child D) and one sibling and the parents of child C have low NK levels. Child D has recurrent viral infections but has had EBV infection in the past without developing HLH. It is tempting to relate child D's infections to her reduced NK numbers but her social circumstances are extremely poor and are certainly a contributing factor. Child C's family have not suffered significant illness.

The relationship of this group's NK deficiency to Fanconi Anaemia is unclear. Child B and Child D have Fanconi mosaicism. Child C had abnormal chromosome stress testing but this was not the classical appearance seen in FA and his parents have normal results. However this lack of familial mosaicism is unhelpful because child B and D's parents also have normal results and we know from previous studies that heterozygotes have normal stress tests (Rosendorff et al, 1988). Child C had some phenotypic features of FA and interestingly child A also has features suggestive of FA. In child A's case a link between NK deficiency and FA was unknown at the time of diagnosis and he was not tested prior to transplant. He will now undergo fibroblast testing as a positive diagnosis would increase his risk of secondary malignancy (CF Chapter 4). Child A did however undergo an unmodified transplant conditioning regimen without ill effect.

All 3 children had pyrexia, hepatosplenomegaly, lymphadenopathy and evidence of bone marrow failure at diagnosis however all had quite dissimilar courses. Child A had a slowly evolving, indolent course and the main reason for his ultimate bone marrow transplant was the increasing incidence of life threatening infections. It could be argued that his HLH responded to immune modulation with CSA and prednisolone but his NK deficiency necessitated bone marrow transplantation. Child C on the other hand had characteristic fulminant disease which was unresponsive to immune modulation and could only have potentially been cured by bone marrow transplantation. The German experience of successful transplant outcome in individuals with active disease (Durken et al, 1999), suggests that in future any child presenting in the Traveller group with these symptoms should undergo urgent BMT. Finding either a sibling donor or familial match in this group may not be a major problem as extended family donors may have identical HLA phenotypes. (*cf* chapter 8). Nevertheless because of time constraints other possibilities may need to be explored (*cf* chapter 6). The demonstration of successful bone marrow transplant using matched unrelated or HLA genetically non-identical donors provides other options when faced with fulminant HLH (Durken et al,

1997). The use of Etoposide containing regimens may offer short term benefit and should be considered whilst awaiting transplant (Hirst et al, 1994; Chen, 1998).

Child B's clinical course was similar to that previously described in viral associated HLH and responded quickly to steroid. He then developed an acute abdominal obstruction related to a lymphoproliferative disease. T-cell lymphoma development has been noted by Chen et al in their 6 year longitudinal study of HLH (Chen et al, 1998). However Chen describes a group of sporadic HLH in whom T-cell clonality was demonstrated. Child B's lymphoproliferation lacked clonality and whilst it also showed the presence of EBV (presumably the aetiological factor) the appearances were those of an immunodeficiency related lymphoproliferation. This may be related to his underlying NK deficiency or perhaps to his Fanconi anaemia (*cf* Cpt 4) (Rosenberg et al, 2003).

Conclusions

These cases illustrate the role of NK deficiency in the development of HLH in this group. In 2 of the 3 cases it is most likely responsible for their subsequent problematic clinical course. It is unclear what role FA has to play in these cases and why individuals with FA develop NK deficiency. It currently appears that only those with absolute NK deficiency are at risk of developing HLH although many of those with FA do have partial NK deficiency. (*cf* chapter 4).

We have identified a group of children from within the Traveller group who are at risk of HLH and another group with partial NK deficiency that may be at risk. It is important to screen all those with FA or who are phenotypically FA (potential FA mosaics) for evidence of NK deficiency. Those who have partial or complete NK deficiency should have further screening for perforin deficiency and PRF1 mutations. They should have regular monitoring and aggressive management of viral infections in an attempt to prevent HLH. Those individuals presenting with evidence of HLH should be investigated as Arico et al suggest (Fig 5.4) and those who show a fulminant course should be considered for immune modulation including Etoposide and early sibling, familial or unrelated bone marrow transplantation.

Table 5.1 Characteristics of NK Cells and other cytolytic effectors.

	NK Cells	T Cells	Monocytes	Neutrophils
Surface Antigens#				
CD2	70-90	>95	<5	<5
CD3	0	>95	0	0
CD8	30-40	30-40	<5	<5
CD11b	80-90	10-15	>90	>90
CD15	<5	<5	60-80	>95
CD16	80-90	<5	10-15*	>95
CD56	>95	<5	<5	<5
Functional attributes				
ADCC	Y	N	Y	Y
NK activity+	Y	N	N	N
Phagocytosis	N	N	Y	Y
Immunologic memory	N	Y	N	N
Proliferative capability	Y	Y	N	N

Legend: #, % of cells from each lineage that express antigen; *, CD16 is also expressed by most cultured or activated monocytes; +, NK activity is spontaneous antibody-independent non-MHC-restricted cytotoxicity. >, greater than; <, less than; ADCC antibody dependant cellular cytotoxicity; Y, yes; N, no.

(Robertson et al, 1990)

Table 5.2 Immunodeficiency with NK cell defects

Primary immunodeficiencies with NK cell deficiency

Severe combined immunodeficiency
Reticular dysgenesis
Common variable immunodeficiency with monocyte dysfunction
Chediak-Higashi syndrome
Leukocyte adhesion deficiency

Other syndromes with NK cell Deficiency

Hodgkin's Disease
Xeroderma pigmentosum
Lymphohistiocytosis
Pre-Leukaemia and Leukaemia
Pure Red cell aplasia
Depression/bereavement
Bloom's syndrome
Acquired immunodeficiency syndrome
Chronic fatigue syndrome

Primary immunodeficiency due to selective NK cell deficiency

Lipinski et al 1980
Four unrelated children with infections, aged 2 to 13, all had decreased interferon γ production; two had partial albinism.

Biron et al 1989
13-y-old girl with severe herpes virus infections

Komiyama et al 1990
12-y-old male with Hodgkin's; 6-y-old brother has respiratory infection and adenopathy.

From Stiehm ER. New and Old Immunodeficiencies. Pediatr Res 1993;33(Suppl) 1;S2-S7

Table 5.3 Diagnostic guidelines for haemophagocytic lymphohistiocytosis

Clinical and laboratory criteria[a]
Fever (≥7 day's duration of ≥38.5°C)
Splenomegaly (≥3cm below costal margin
Cytopenia (≥2 of 3 lineages in peripheral blood and not caused by hypocellular or dysplastic bone marrow)
Haemoglobin (<9.0g/dl)
Platelets (<100x10^9/l)
Neutrophils (<1.0x10^9/l)
Hypertriglyceridaemia and/or hypofibrinogenaemia
Fasting triglycerides ≥ 3 SD above normal average for age
Fibrinogen ≤3 SD below normal average for age
Histopathologic criteria[b]
Haemophagocytosis in any of the following
Bone marrow
Spleen
Lymph node
No evidence of malignancy

[a] as proposed by Henter et al (Henter JI, Elinder G, Ost A. FHL Study Group of the Histiocyte Society. Diagnostic guidelines for Haemophagocytic lymphohistiocytosis. Semin Oncol 1991;18:29-33)

[b]If haemophagocytic activity is not found at presentation, continued search for haemophagocytosis is indicated (spleen, lymph nodes and serial bone marrows). If no family history, diagnosis of haemophagocytic lymphohistiocytosis (HLH). If family history of HLH, diagnosis of familial haemophagocytic lymphohistiocytosis (FHL). Parental consanguinity strongly suggests FHL (Egeler RM, Shapiro R, Loechelt B, Filipovich A. Characteristic immune abnormalities in haemophagocytic lymphohistiocytosis. J Pediatr Hematol/Oncol 1996. 18(4):340-345)

Table 5.4 Child C Laboratory results at presentation

Test	Result	Normal Values
Hb (g/dl)	8.3	11.5-14.5
Platelets (x10^9/l)	154	150-450
White cells (x10^9/l)	2.79	5-15
Neutrophils (x10^9/l)	1.9	1.5-8.5
PT (seconds)	21.8	13-14.4
APTT (seconds)	48.9	27.1-38.9
Fibrinogen (g/l)	1.3	1.5-4.0
Bilirublin (μmol/l)	86	2-14
ALT (u/l)	87	≤35
Alkaline phosphatase (u/l)	1560	100-300
LDH (u/l)	1576	310-790
Albumin (g/l)	18	36-50

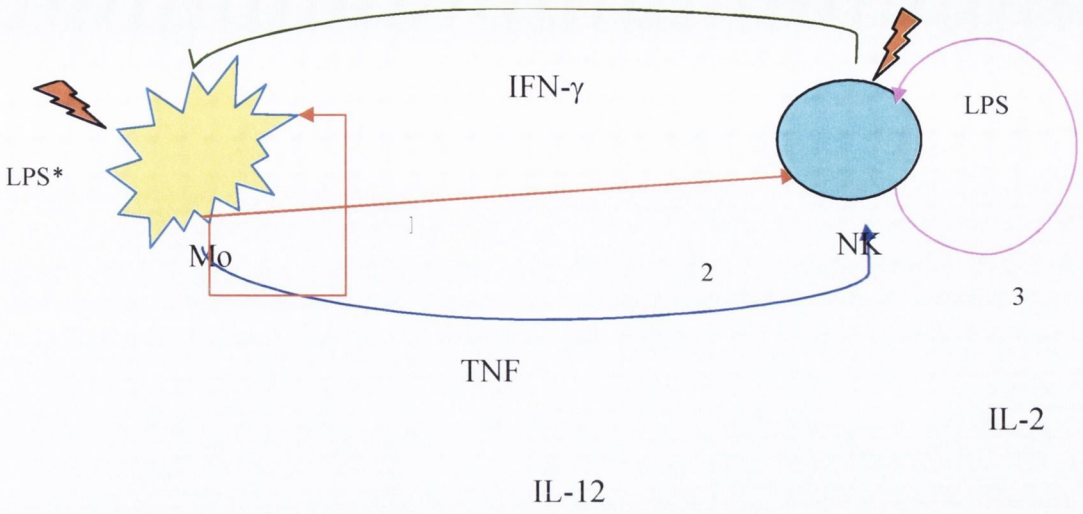
Key: PT, prothrombin time; APTT, activated partial thromboplastin time; ALT, alanine transferase; LDH, lactate dehydrogenase; g/dl, grams per decilitre; g/l, grams per litre; μmol/l, micromoles per litre; u/l, units per litre; ≤ less than or equal to.

Table 5.5 Results of immune function assays Child C (Taken at initial presentation)

Total lymphocyte Count 683 (5500-8500) x 10^9/l

T cells (CD3)	79% (62-69)	473 (1800-3000) x10^9/l
Helper TC (CD3/4)	47% (30-40)	545 (1000-1800) x10^9/l
Suppressor TC (CD3/8)	18% (25-32)	211 (800-1500) x10^9/l
B Cells (CD19)	5% (21-28)	10 (700-1300) x10^9/l
NK cells (CD16/56)	0% (8-15)	1 (200-600) x10^9/l

Fig 5.1 Schematic diagram of NK monocyte interaction



Legend; 1 = positive feed back for IL-12
2, positive feed back IFN-γ
3, increased NK proliferation

Mo, monocyte; TNF, tumour necrosis factor, IFN-γ, Interferon gamma; LPS*, Lipopolysaccaride

* Lipopolysaccaride is found on the outer cell wall of gram negative bacteria.
It is shed by the bacteria and can bind to receptors on the surface of
primed macrophages and NK cells, increasing phagocytosis and cytokine production

Fig 5.2 Pathways of Lymphocyte mediated cytotoxicity

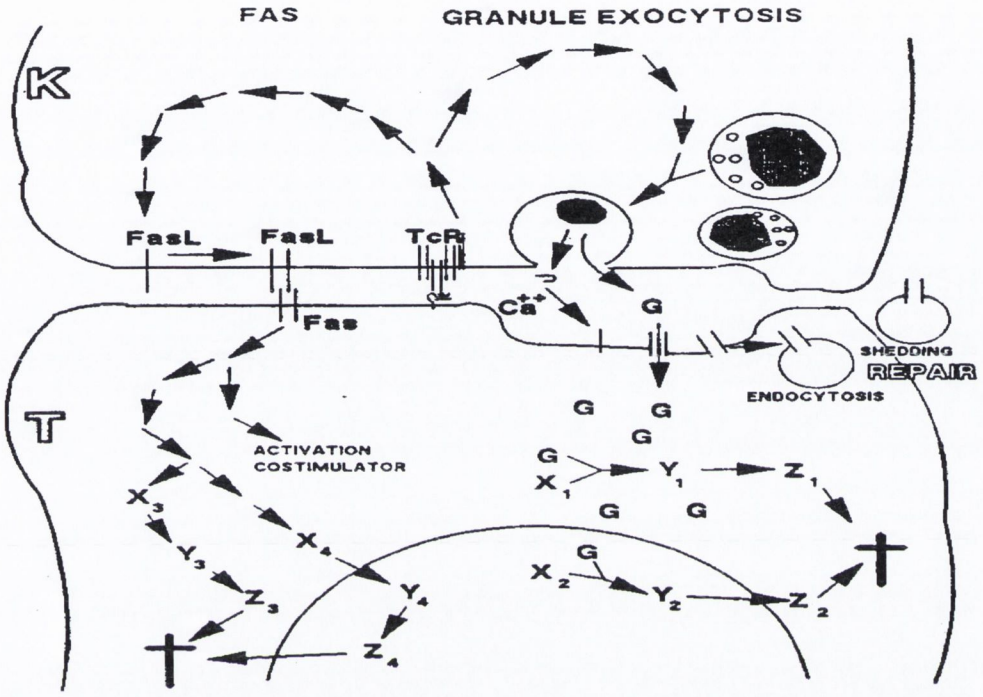


Figure 1. Pathways of Lymphocyte-Mediated Cytotoxicity

The diagram shows a CTL utilizing the two defined cytotoxicity pathways. On the right is the granule exocytosis pathway, consisting of a modified classical receptor-triggered degranulation pathway using preexisting secretory granules in the killer cell (K). The act of target cell binding engages the TCR or other triggering receptor, which rapidly induces a polarization in the cytoplasm of the effector cell, setting up a highly directed degranulation into the synapse-like gap between the two cells. From the granule core, the water soluble cytolysin/perforin molecules (sideways J) dissolve and undergo a Ca-triggered conformational change, rendering them amphitropic (straight) so that they insert into lipid bilayers. There they aggregate and form pores capable of passing both ions and macromolecules, including granzymes (G). The pore-damaged membrane is repaired by endocytosis or shedding, but the granzymes enter the cytoplasm, where their physiological target with regard to cell death is still unknown. Two possibilities are shown: X_1–Y_1–Z_1, which is independent of the nucleus, and X_2–Y_2–Z_2, in which nuclear damage results in cell death. X_2 may be $p34^{cdc2}$ kinase.

The left side shows the Fas pathway, in which TCR signaling again induces polarization but also induces up-regulation of Fas ligand expression. This, in turn, cross-links Fas on the target cell. This may not lead to cell death (first branch), or may lead to death by a nucleus-independent route (X_3–Y_3–Z_3) or as a result of nuclear damage (X_4–Y_4–Z_4).

(From Henkart PA, Immunity 1994;1:343-346)

Fig 5.3 Proposed pathway for diagnosis of HLH (Arico et al, 2002)

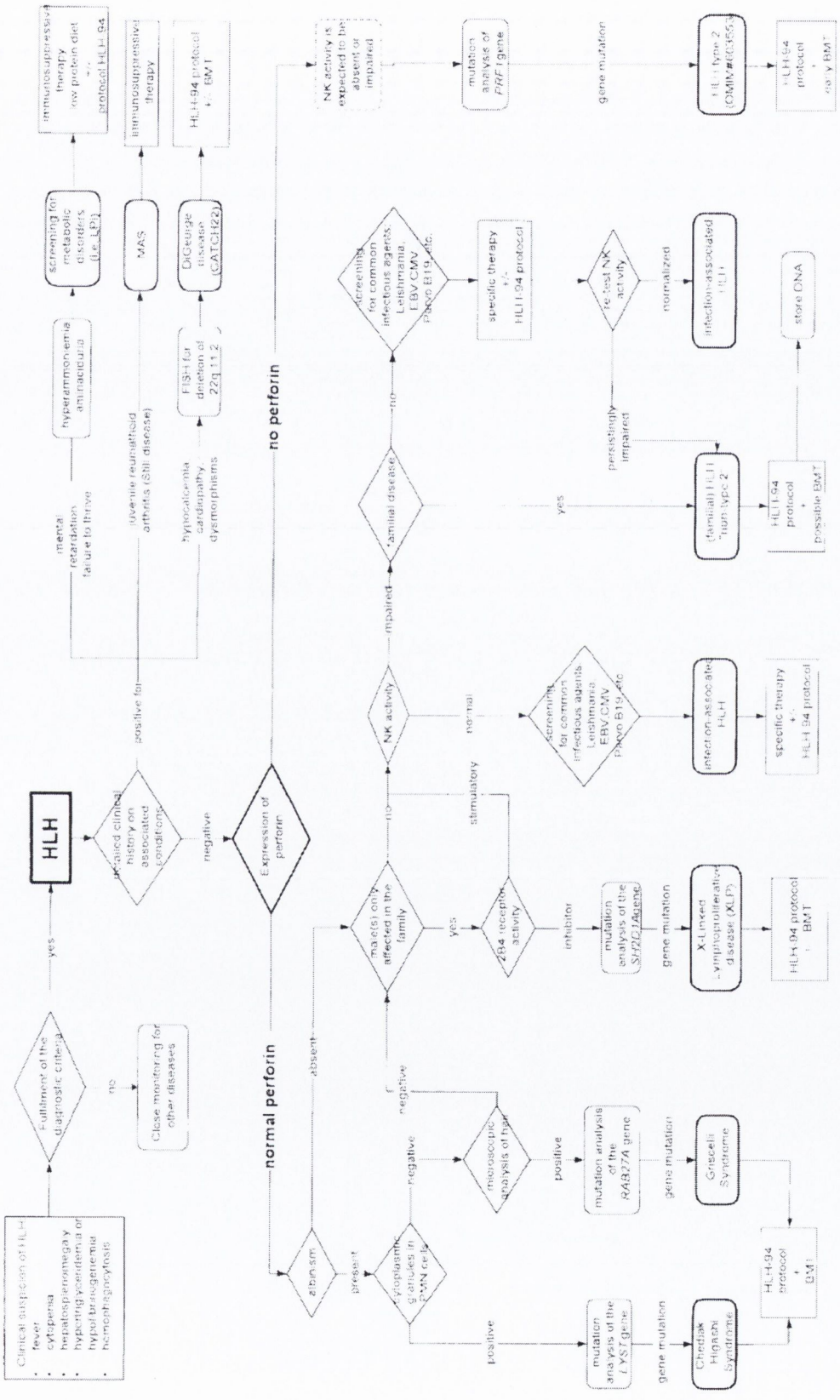
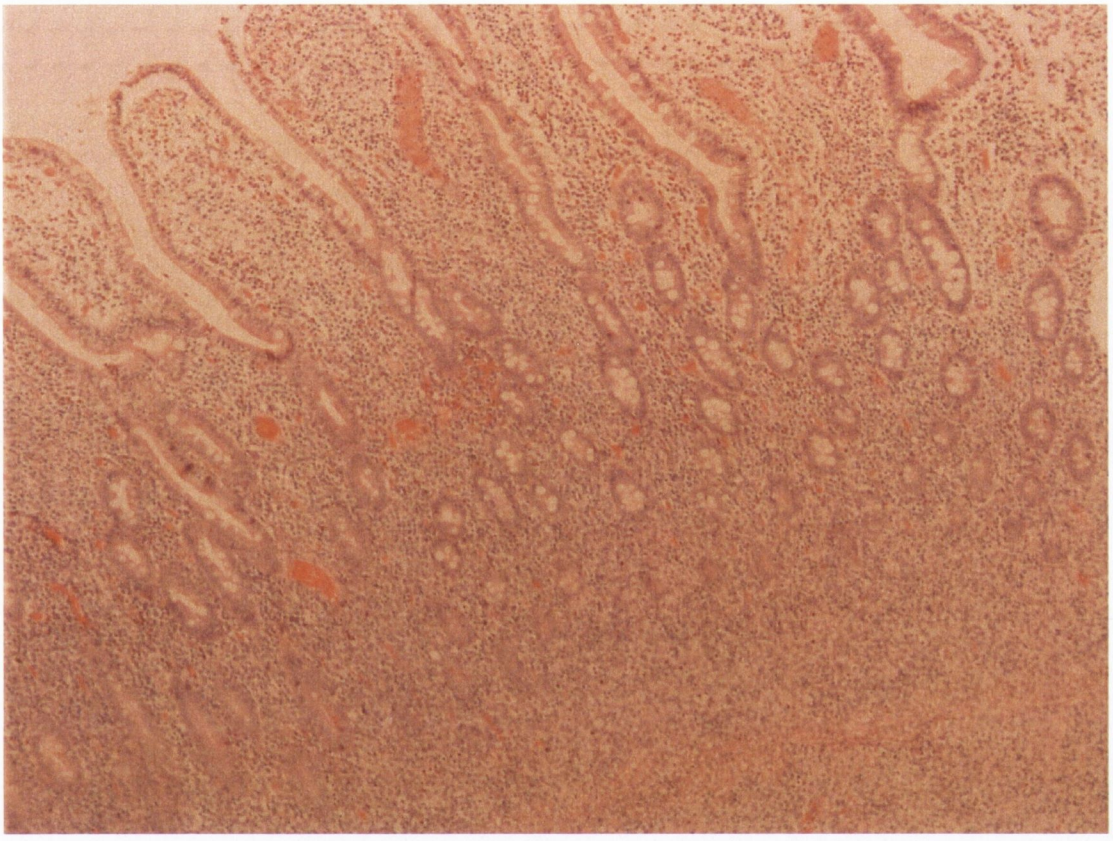


Fig. 1. Proposed diagnostic approach to haemophagocytic lymphohistiocytosis. All patients in whom the diagnostic criteria for HLH are fulfilled undergo evaluation of perforin expression by flow cytometry. Male patients in whom perforin expression is normal are evaluated for the behaviour of the lymphocyte 2B4 receptor. Natural killer activity is then used to discriminate between other genetic and possible infection-associated forms of HLH.

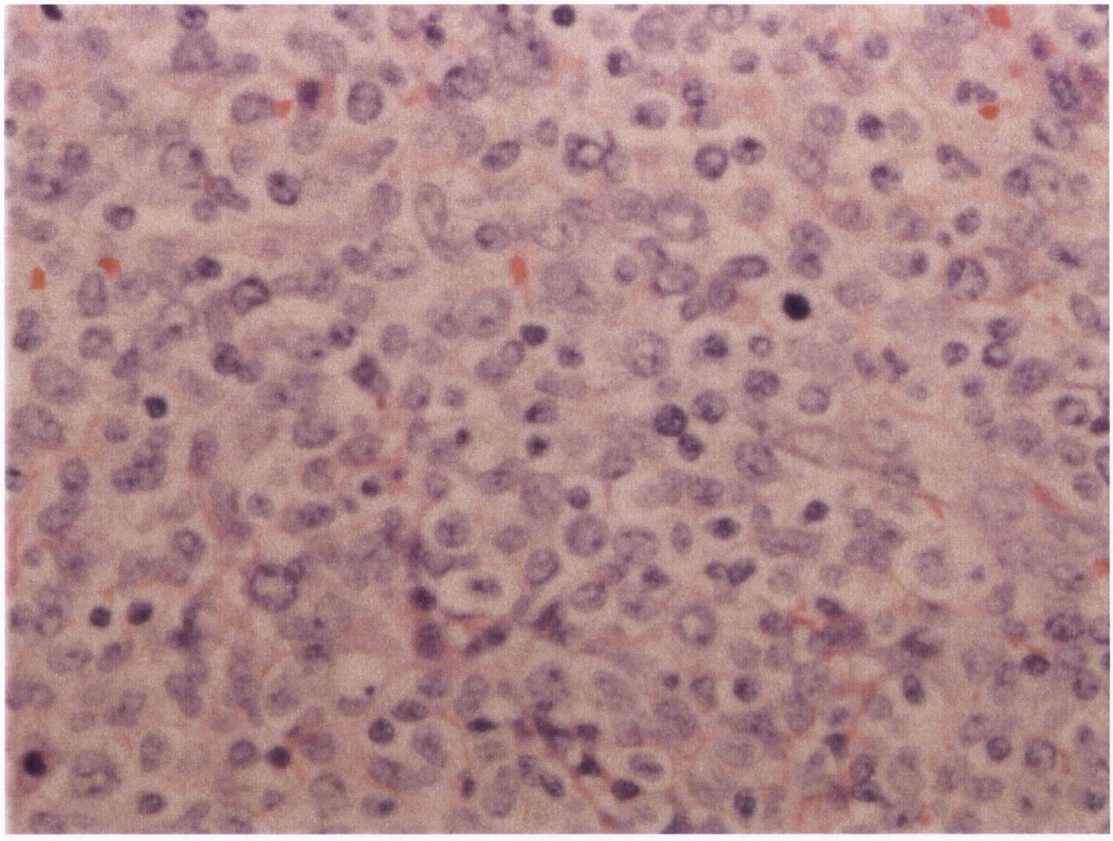
Fig 5.4 Ileal resection Child B showing a dense infiltrate of large atypical cells involving submucosa, muscularis mucosa and lamina propria.



(Photographed by Dr M Jeffers, Consultant Histopathologist, AMNCH, Tallaght)

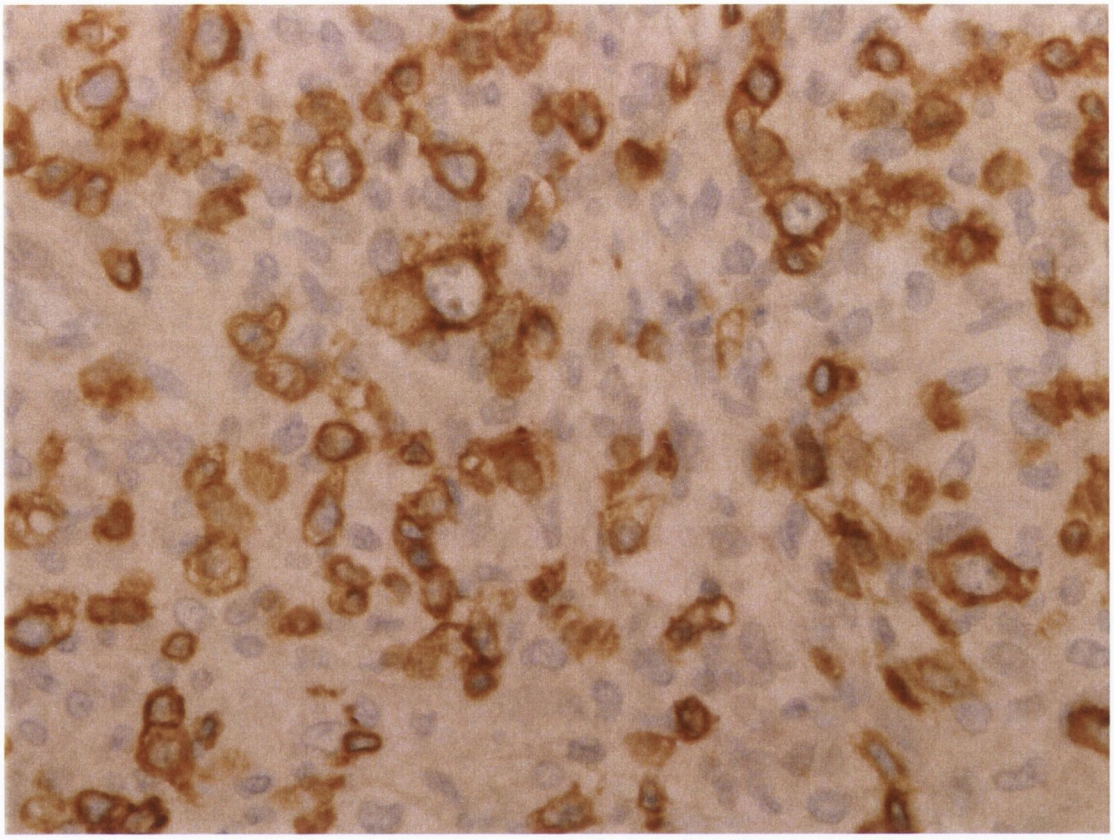
Fig 5.5 High power view of atypical cells

Total replacement by a uniform population of large cells with abundant cytoplasm.



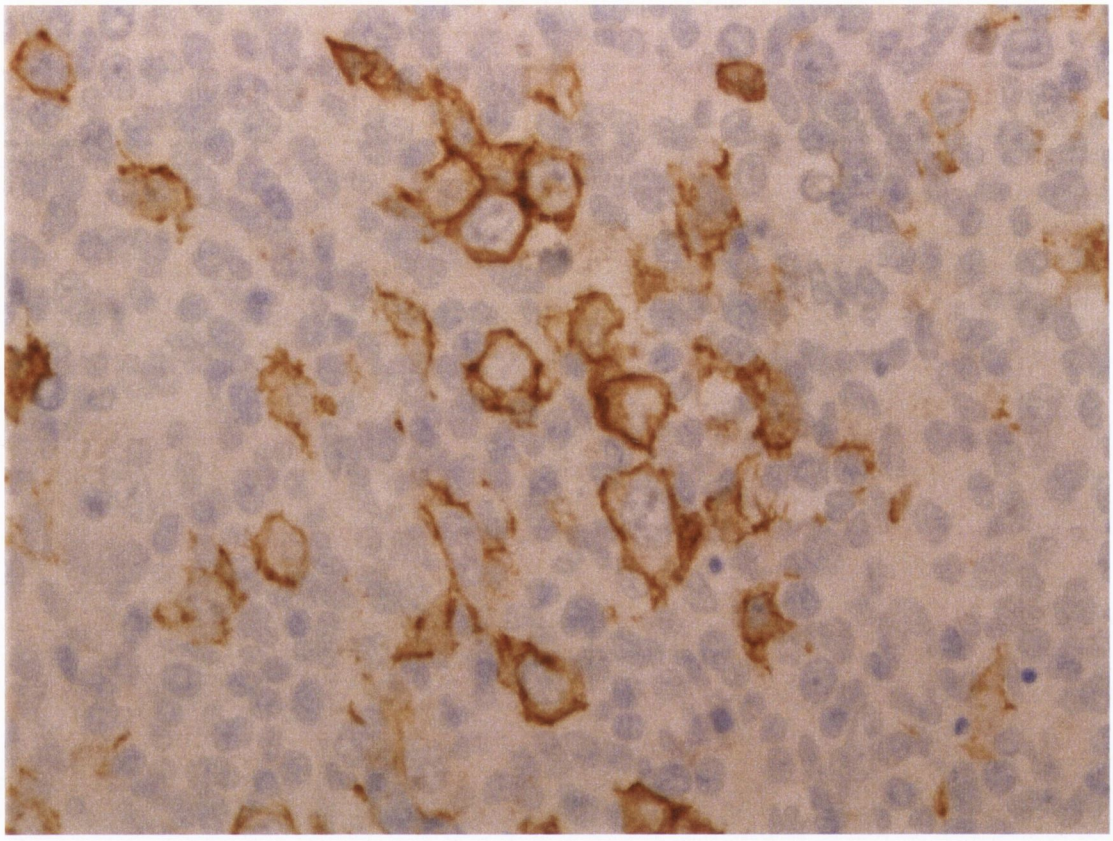
(Photographed by Dr M Jeffers, Consultant Histopathologist, AMNCH, Tallaght)

Fig 5.6 CD3 T cell expression of atypical cells (Brown staining of cytoplasm)



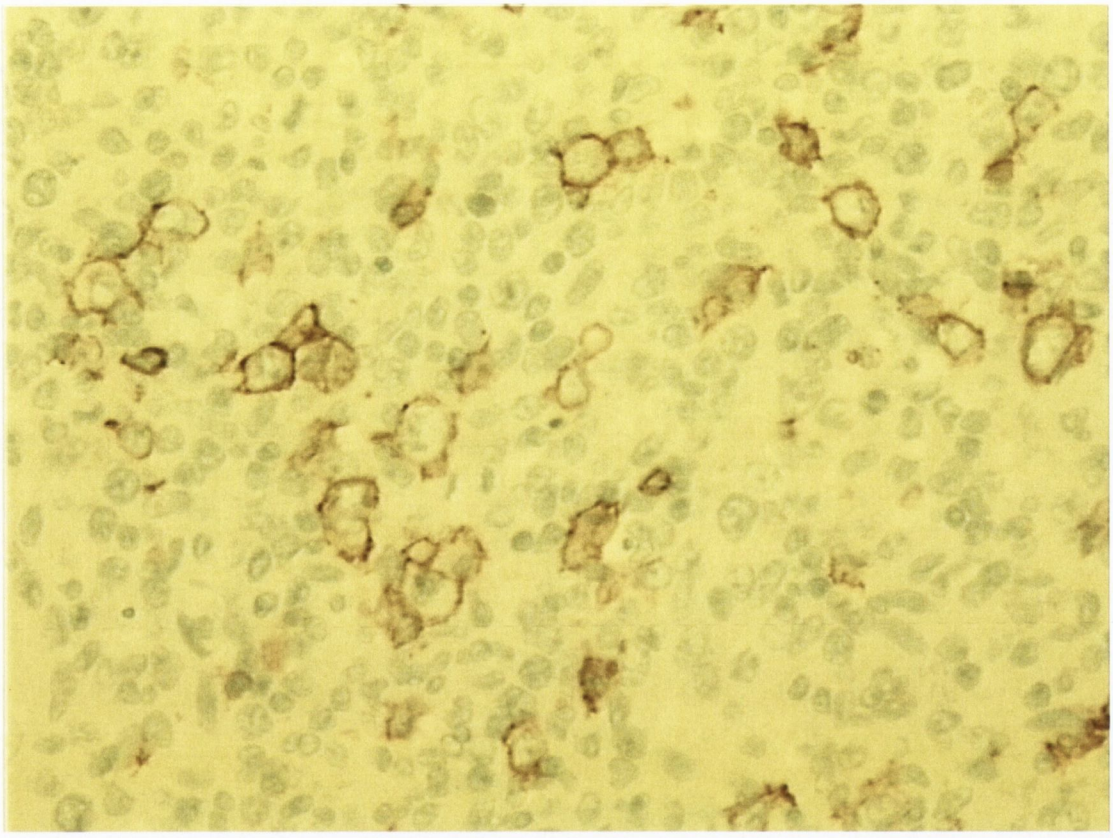
(Photographed by Dr M Jeffers, Consultant Histopathologist, AMNCH, Tallaght)

Fig 5.7 CD20 B cell expression of atypical cells. (Brown staining of cytoplasm)



(Photographed by Dr M Jeffers, Consultant Histopathologist, AMNCH, Tallaght)

Fig 5.8 Epstein Barr Protein expression (Positive cytoplasmic staining)



(Photographed by Dr M Jeffers, Consultant Histopathologist, AMNCH, Tallaght)

Fig 5.9 Bone Marrow Haemophagocytosis (x60) from Child C

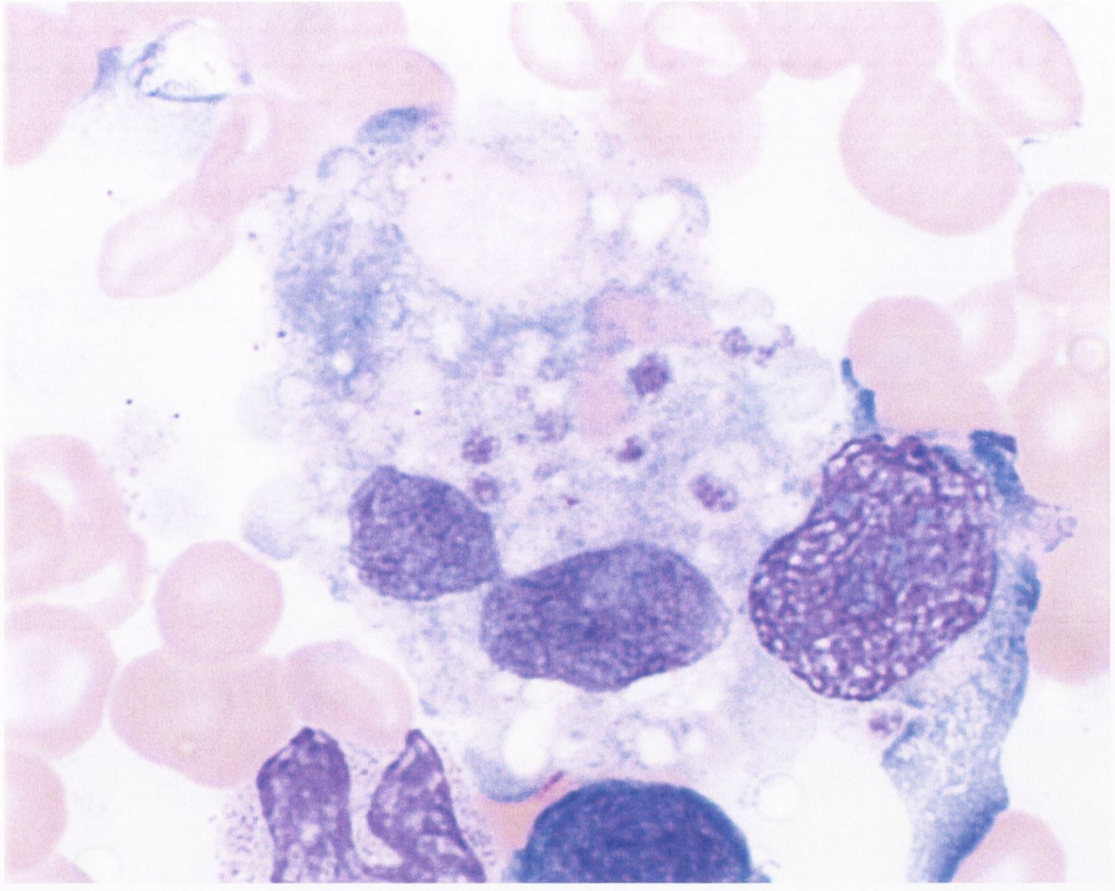


Fig 5.10 Bone Marrow histiocytes with erythrocyte inclusion (x100) from child C

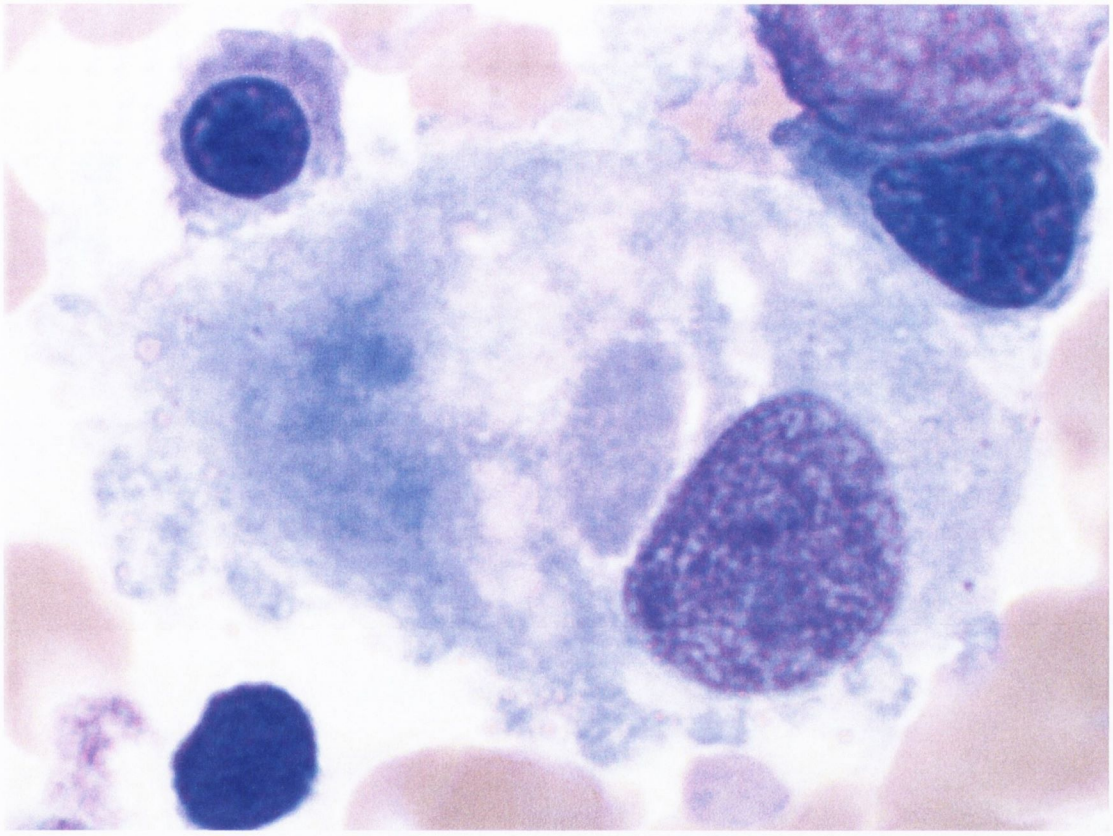
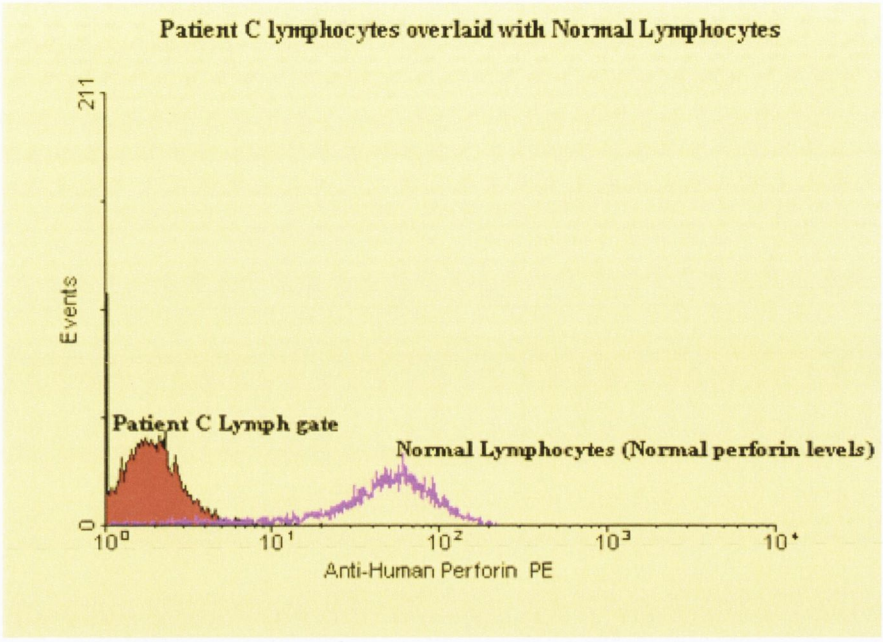


Fig 5.11.Perforin Analysis Child C demonstrates absence of Perforin expression



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Chapter 6

Hurler syndrome

The Mucopolysaccaridoses

The mucopolysaccaridoses are a group of inherited metabolic disorders caused by a deficiency of lysosomal enzymes that are needed for the degradation of glycosaminoglycans (GAGs, also known by the older name mucopolysaccarides). These enzyme deficiencies are characterised by an accumulation of lysosomal GAGs, excessive urinary excretion of GAGs, progressive physical and mental deterioration and in its most severe form premature death (Muenzer J, 2000). Each type of mucopolysaccharidosis has a specific lysosomal enzyme deficiency and is associated with a characteristic degree of organ involvement and rate of deterioration (Table 6.1), leading to the accumulation of dermatan sulphate, heparin sulphate, keratan sulphate or a combination. This ultimately leads to cell, tissue or organ dysfunction. The mucopolysaccaridoses (MPSs) share many clinical features including; organomegaly, coarse abnormal features, abnormal joint mobility, and hearing, vision, upper airway and cardiovascular dysfunction. Neurological manifestations and mental retardation predominate in MPS II and MPS III, appearing early in the course of the disease and in MPS IH where they are delayed until after the skeletal abnormalities become obvious. The world wide incidence of MPS is between 1:50,000 and 1:100000 live births (Danos et al, 1995), but the incidence in Northern Ireland is estimated to be 1:25,000 live births. The Northern Ireland data was further analysed to calculate the incidence of each of the MPSs. The incidence of MPS IH was 1:76,000; 1:280,000 for MPS IH/S; 1:140,000 (1:72,000 live male births) for MPS II; 1:280,000 for MPS III and 1:76,000 for MPS IVA. No cases of MPS IS, IVB or MPSVI were found (Nelson, 1997).

Clinical features of MPS-1H (Hurler syndrome)

The majority of individuals with MPS-1H have a normal appearance at birth with only slight developmental delay noted in the first 6 months. However physical examination often reveals hepatosplenomegaly, nasal discharge and noisy breathing. Facial features

coarsen within the 1st year. Generally the head is large with a depressed nasal bridge and flat nose. Dysostosis multiplex is obvious at birth. Patients are very short with growth stopping at 2-3 years of age. The disease progresses rapidly during the first 2-4 years with patients becoming less mobile from progressive stiffening of the joints. Chronic airway obstruction, respiratory infections and cardiac complications in the mitral valves from storage of mucopolysaccarides become prominent features of the disease and often cause death (Hopwood et al, 1993). Obstructive sleep apnoea is almost universal and whilst it may initially benefit from adenotonsillectomy, later in the disease nocturnal O2 therapy may be required (Wraith, 1995). Patients with Hurler syndrome have delayed speech development and severe mental handicap associated with extensive neuronal abnormalities. They reach a peak of intellectual function at about 2 years or earlier, after which there is a steady regression (Hopwood, 1993).

Skeletal abnormalities (Dysostosis Multiplex) are invariably found and cause progressive disability. The odontoid process of C2 is one of the most clinically significant skeletal areas to be affected by accumulation of glycosaminoglycans. Bony dysplasia of the dens has been observed in many patients. Lack of normal odontoid ossification has been associated with C1-3 subluxation. Accumulation of GAGs in the dural structures and paraspinal ligaments produces additional potential morbidity with the possibility of cervical cord injury secondary to extrinsic compressive myelopathy (Hite et al, 2000). It has been suggested that the incidence of odontoid dysplasia can be as high as 94% and that 38% of patients demonstrate anterior C1-C2 subluxation (Belani et al, 1993).

Other orthopaedic problems include acetabular dysplasia, kyphoscoliosis, carpal tunnel syndrome, trigger fingers and genu valgum. Genu valgum develops in the first 2-5 years of life (Odunusi et al, 1999). Carpal Tunnel syndrome causes sensory and motor signs which can limit hand function by causing "clawing". It has been suggested that subtle signs of carpal tunnel syndrome can be seen as early as 2 years although the children are asymptomatic. Attention should be paid to alterations in grasp and increasing difficulty with fine motor tasks and manual clumsiness. Nerve conduction studies are always abnormal and early intervention leads to improved outcome (Haddad et al, 1997; Van Heest et al, 1998).

Genetics

Hurler syndrome is an autosomal recessive condition (Clarke et al, 1997). The gene encoding a-iduronidase has been localised to chromosome 4p16.3 and spans 19 kilobases and includes 14 exons (Scott et al, 1995). There are currently at least 63 defined mutations that include 13 nonsense mutations and 27 missense mutations (Scott et al, 1995; Beesley et al, 2001). The nonsense mutations, which include 2 major alleles W402X and Q70X, result in a total lack of enzyme function and produce a severe phenotype in homozygotes and when associated with heterozygosity for other severe mutations (Scott et al, 1995). The frequency of Q70X ranges from 7% in Britain to 65% in Scandinavia whilst W402X is found in 52% of British patients with MPS-I and has an even higher frequency in Irish MPS-I patients suggesting an Anglo-Saxon or Celtic origin (Scott et al, 1995). Genotype-phenotype analysis has demonstrated that only low levels of residual enzyme activity are required to protect against severe disease (Scott at al, 1995).

Pathophysiology

Most of the digestive process in the human cell takes place in the lysosome which contains more than 40 enzymes, many of which are acid hydrolases. The acid pH maintained within the lysosome activates enzymes that then degrade proteins, nucleic acids and complex sugars. Glycosaminoglycans (GAG) are continuously produced by the normal turnover of proteoglycans in connective tissues. Their stepwise degradation in the lysosome involves 4 glycosidases, 5 sulfatases and one non-hydrolytic transferase. In MPS because of enzyme dysfunction most of the cells in the organism are clogged with undigested intermediates (Danos et al, 1995).

A recently developed IDUA knock out mouse gives some insight into the evolution of Hurler syndrome. The mouse appears normal at birth but increased lysosomal storage has occurred in the macrophage system, the chondrocytes within the articular surfaces of bone and in the trachea although there is little hepatocyte or neuronal storage. By eight weeks 15-25% of hepatocytes are affected, the splenic sinusoids are swollen and cytoplasmic vacuolation is found in the purkinje cells of the cerebellum, neurons of the

cerebral cortex, glial cells and cells of the leptomeninges. Skeletal abnormalities are obvious by 3 weeks of age with flattening of the facial profile, thickening of facial bones and the digits and anterior flaring of the ribs and extensive dysostosis is detectable by 15 weeks of age (Clarke et al, 1997).

In humans MRI scanning has shown radially oriented cystic lesions in the centrum semiovale, peritrigonal white matter, corpus callosum and pericallosal region which correspond to perivascular lacunae that are seen histologically. MRI abnormalities are seen even when the CT scan is normal (Afifi et al, 1990). Progressive cervical myelopathy due to soft tissue compression of the spinal cord due to mucopolysaccharide deposition has been reported even following BMT causing tetraparesis but which was partially corrected by laminectomy and decompression with duroplasty (Kachur et al, 2000).

Progressive ophthalmologic findings include corneal clouding, retinal pigmentary degeneration and optic nerve atrophy. These abnormalities are caused by intracellular deposition of dermatan and heparin sulphate (Gullingsrud et al, 1998).

The cause of carpal tunnel syndrome appears to be a thickened flexor retinaculum and abnormal tenosynovium which engulfs the flexor tendons and restricts single tendon and differential motion. Histologically the flexor retinaculum and tenosynovium and epineurium contain numerous foamy macrophages and fibroblasts containing empty membrane bound vacuoles in a loose connective tissue matrix (Haddad et al, 1997). Carpal tunnel syndrome appears to progress despite bone marrow transplantation.

Abnormalities have also been noted in the growth giving rise to a disproportionate short trunk dwarfism. Abnormalities include irregularities in chrondrocyte orientation within the growth plate along with disruption of the normal columnar architecture. Vacuolization with enlargement of the cellular border is the characteristic ultrastructural finding (Silveri et al, 1991).

Cardiomyopathy and cardiac arrhythmia are leading causes of death in untreated cases of hurler syndrome. A recent study of 64 patients found that the most common lesion was thickening of the mitral valve often in association with regurgitation or stenosis.

Aortic valve thickening and asymmetric septal hypertrophy or hypertrophic cardiomyopathy was less common (Dangel, 1998). In earlier cardiac studies, structural examination had revealed infiltration of the coronary arteries, heart valves, mural endocardium, myocardial walls and aorta by large vacuolated cells (Hurler cells) and intracellular and pericellular accumulation of dermatan and heparin sulphate containing glycosaminoglycans and glycolipids. In addition there was a heavy deposit of collagen in the cardiovascular system (Renteria et al, 1976). It has recently been suggested that intracellular accumulation of dermatan sulphate in the fibroblasts of patients with hurler's syndrome disrupts normal elastogenesis and contributes to a loss of elastin in the skin, tendons, cardiac valvular apparatus and coronary artery walls producing the characteristic appearances. It has been postulated that elastic fibrils found in limb buds and perichondrial tissue may be crucial factor in maintaining the proper shape of the embryonal skeleton, and disruption of these fibrils may contribute to the skeletal deformities found in Hurlers (Hinek et al, 2000).

Diagnosis

Analysis of urinary GAGs was the first method of diagnosis of MPS and remains a useful initial diagnostic test (Constantopoulos et al, 1975; Cohen et al, 1977). Any child who is suspected of having a MPS based on clinical appearance, radiological features or urinary GAGs screening tests should have a definitive diagnosis established by enzyme assay. Serum or fibroblasts (Taylor et al, 1991) can be used but normally leucocyte analysis is employed (Omura et al, 1976). Recently it has been suggested that dried blood spots could be used as a rapid screening technique to detect those with lysosomal disease (Chamoles et al, 2001).

Therapeutic strategies for MPS 1H

Haematopoietic stem cell Transplantation. (HSCT)

Haematopoietic stem cell transplantation has resulted in significant clinical improvement of somatic disease in Hurler syndrome and increased long term survival.

Resolution or improvements have occurred in hepatosplenomegaly, joint stiffness, facial appearance, obstructive sleep apnoea, heart disease, communicating hydrocephalus and hearing loss (Peters et al, 1998a). In contrast skeletal and ocular abnormalities are not corrected and may progressively worsen although there is some evidence that there may be a beneficial effect on upper limb joint mobility (Gullingsrud et al, 1998; Krivit et al, 1999; Weisstein et al, 2004). Orthopaedic procedures including femoral osteotomies, acetabular reconstruction and posterior spinal fusion have been necessary following transplantation.

Patients who were transplanted before 24 months of age and who had a baseline mental development index greater than 70 had an improved, sometimes normal, developmental outcome but in most children neuropsychological outcome is not normal (Peters et al, 1998b). Donor selection can be a problem with many children lacking a suitable sibling donor but similar outcomes were demonstrated using unrelated donors. One of the main problems with matched unrelated transplant (MUD) has been an increased incidence of graft failure. This appears to be related to the bone marrow cell dose infused, with those receiving a dose more than 3.5x10^8 cells/kg having better engraftment. (Peters et al, 1996) Ultimate neuropsychological function was significantly lower when the donor was a carrier or when the donor was normal but the recipient's engraftment was incomplete resulting in lower than normal enzyme levels (Peters et al, 1998a). Matched or partially matched unrelated cord-blood transplants have recently been successfully used to treat Hurler syndrome and may provide options for those without a suitable family donor (Staba et al, 2004).

Attempts have been made to improve somatic and neurological outcome by using a booster infusion of allogeneic multipotential bone marrow–derived mesenchymal stem cells in patients who had previously undergone successful allogeneic bone marrow transplantation. This strategy has had some success but further trials are necessary to define the role of such therapy in the management of Hurler syndrome (Koc et al, 2002).

Gene Therapy

Gene therapy is an attractive option but the vehicle for gene transfer remains problematic. Experiments using genetically modified autologous bone marrow stem cells which express iduronidase have not resulted in clinical improvement in canine alpha–L–iduronidase deficiency even when injected in pre-immune fetal pups (Lutzko et al, 1999). Earlier reports of in vitro correction of Hurler syndrome using a retroviral vector transfer to primitive CD34+ stem cells by Fairbairn et al has not as yet brought in vivo success (Fairbairn et al, 1996). Baxter et al have shown that in vitro retroviral transduction into bone marrow derived mesenchymal stem cells has normalised GAG storage in a MPS-IH cell line (Baxter et al, 2002). A team from the Washington University School of Medicine has recently reported prevention of cardiac, bone and auditory and visual MPS manifestations using a retroviral vector expressing canine α-L-iduronidase when given to MPS-1 mice in the newborn period (Liu et al, 2005), and a French team has prevented MPS 1 pathology in a mouse brain using a single injection of gene transfer vectors from adeno-associated virus serotype 5 (AAV5) (Desmaris et al, 2004).

Enzyme Replacement therapy

Enzyme replacement using recombinant enzyme produced in cultured mammalian cells shows promise in reversing the somatic features of MPS. It has been used successfully in dogs and this has supported clinical trials of use in patients with Hurler syndrome. Phase I/II and phase III human clinical trials demonstrated efficacy in treating patients with attenuated forms of MPS-I (i.e. with little or no neuronal involvement) (Brooks, 2002; Wraith, 2001). An extensive evaluation was undertaken by Kakkis et al who monitored the effect of a-L-iduronidase over 52 weeks on patients aged 5-22 years with MPS I H-S. This showed an improvement on hepatosplenomegaly, growth and mobility but it was not without side effects, in particular recurrent urticaria (Kakkis et al, 2001). A phase III randomised, double blinded multinational study of 45 patients who received 100U/kg weekly for 26 weeks demonstrated improved Forced Vital capacity (FVC), 6-minute walk distance, reduced hepatomegaly and urinary GAG and in more severely affected patients improved sleep apnoea/hypopnea and shoulder flexion when compared with placebo group (Wraith et al, 2004). More recently enzyme replacement therapy

was used before and after haematopoietic stem cell transplantation and whilst it is too early to determine if it will improve the long-term outcome following HSCT, the combination was well tolerated (Grewal et al, 2005). It does not appear that intravenous enzyme replacement adequately reverses the effects of storage disease on the central nervous system, possibly because the blood –brain barrier alters enzyme distribution. It has been shown that weekly intrathecal therapy can reduce mean total brain GAG levels to normal and reduce meningeal GAG levels by 57% in MPS-I affected dogs (Kakkis et al, 2004). The exact role that enzyme replacement has to play in the management of Hurler syndrome remains to be fully elucidated and further prospective clinical trails are needed.

Other Novel Therapies

An interesting and novel approach has been suggested by Keeling et al for those patients with the IDUA stop mutations Q70W and W402X. Gentamicin has been shown to mediate the suppression of stop codons by binding to the decoding site of the small sub-unit rRNA. The addition of gentamicin to cultured Hurler fibroblasts restores 2.5-3% a-L-iduronidase activity with concomitant reduction in GAG storage suggesting a possible role in therapy particularly if it can cross the blood brain barrier (Keeling et al, 2001).

Supportive Therapy

Haematopoietic stem cell transplantation does not preserve intelligence in severe MPS II or MPS III and therefore is not recommended for any MPS except Hurler syndrome. Many children with Hurler syndrome lack suitable donors and even those who do undergo transplantation have only partial resolution of their disease. Supportive measures with particular attention to cardiovascular and respiratory complications, hearing loss, carpal tunnel syndrome, spinal cord compression and hydrocephalus can greatly improve the quality of life for these patients and their families. Psychological support and special attention to their educational needs such as the provision of special school places is also of benefit.

Patients Materials and methods

Patient demographics.

An analysis of the Human Leucocyte antigen (HLA) typing requests made to the Irish Blood Transfusion Service (IBTS) from 1984-1999 was undertaken to determine the number of requests for children with Hurler syndrome and the success of finding a family donor for these children. Thirty one children, 20 boys and 11 girls, from 7 inter-related family groups were diagnosed with Hurler syndrome during this time. Thirteen children came from 5 families. One family had 4 siblings affected; another 3 siblings and the remaining 3 families had 2 affected siblings.

The records of the National Paediatric Transplant Centre, Our Lady's Hospital for Sick Children Crumlin (OLHSC) from 1988-Feb 2002 were then analysed to determine how many of the children identified by IBS records had undergone bone marrow transplantation. Bone Marrow transplantation for Hurler syndrome prior to 1988 was undertaken in specialist centres in the UK, but it was sometimes possible to determine from family histories, the outcome of these early transplants and some, at least, of these children had subsequent long term follow up at OLHSC. The influence of bone marrow transplant on this condition was determined by analysis of nature of the children's disabilities pre and post transplant, the type of transplant received, and the associated complications of the transplant process.

Diagnosis and evaluation.

Ten children had investigation initiated because of a previous family history of Hurler syndrome and the others were investigated because they were noted to have dysmorphic features. One child presented with a gibbus, another with talipes and failure to thrive and 2 had developmental delay. Initial screening was undertaken by demonstrating increased excretion of urinary glycosaminoglycans. A definitive diagnosis was then made by estimation of serum Alpha-iduronidase levels. Gene analysis was undertaken at the Willink Biochemical Genetics Unit, Royal Manchester Children's Hospital. The children's clinical review included skeletal survey, opthalmological and Audiology

review, cardiac assessment and psychological evaluation. HLA typing was undertaken on the affected child, their parents and siblings and in some cases extended family members (*cf* Chpt 8). Recently those without a haploidentical or sibling donor had an unrelated donor search performed. Family members who were potential bone marrow donors had α-iduronidase levels measured before donation to determine if they had normal a-iduronidase levels or if they were heterozygotes for the disease.

Bone Marrow Transplantation

Busulphan and cyclophosphamide was the conditioning regimen for all of the bone marrow transplants. Prior to August 1993 the dose of Busulphan was 4mg/kg/day administered orally for 4 days and followed by intravenous cyclophosphamide 50mg/kg/day given over 1 hr each day for 4 days. This regimen was subsequently adjusted and now Busulphan 5mg/kg/day for 4 days and cyclophosphamide 60mg/kg/day for 4 days is given. Mesna is administered 3 hourly for the duration of Cyclophosphamide therapy. Those who received second transplants prior to 1993 were conditioned using Busulphan 5mg/kg/day x 4 days and cyclophosphamide 50mg/kg/day x 4 days whilst those receiving second transplants since then have been reconditioned using the same regimen as that given for 1st transplants. Anticonvulsant therapy is administered from day -10 to day -5. Initially Phenytoin was the medication used was but in 1994 this was changed to clonazapam 0.1mg/kg/24hours as a continuous infusion because of uncertain busulphan pharmacokinetics when using phenytoin. Cyclosporin A, 1.5mg/kg iv from day -1, is given as graft versus host prophylaxis and the dose adjusted to maintain a level of 100-200 ? units. Urosdiol 10mg/kg is commenced at day -7 as prophylaxis against veno-occlusive disease of liver.

Engraftment is determined by restoration of normal haematopoiesis and the assessment of chimerism studies in association with evaluation of enzyme levels

Follow up.

The children are seen initially every week and thereafter at increasing intervals until they only attend for yearly review. A comprehensive care programme is instituted for

each child, consisting of medical, orthopaedic and ophthalmic review. Regular audiology assessment and psychometric testing is also undertaken.

Results

Analysis of data from the IBTS HLA typing data base and the records of the National Paediatric Bone Marrow Transplant Centre Crumlin suggests that 31 traveller children with Hurler Syndrome were diagnosed between 1984 and February 2002. Twenty children, 11 boys and 9 girls, underwent bone marrow transplantation during this time. Three children were transplanted at centres in the UK, one of whom died within 3 weeks of respiratory failure. The other 17 children were transplanted at the National Bone Marrow Transplant centre, Our Lady's Hospital for Sick Children Crumlin. Six children identified as having Hurler syndrome and who did not undergo transplantation have died. There is no further information available on another 5 children who are listed in the IBS records as having Hurler syndrome. It seems likely that 2 did not undergo transplantation as no family donor was identified. Three others did have family donors, but I can find no record of them at the National Transplant centre. It is possible that they may also have died following transplantation in the UK.

Data is available on 19 transplanted children, including 2 transplanted in the UK, who now attend OLHSC for post BMT monitoring.

The children were diagnosed as having Hurler syndrome between 1 week and 10 months, median 4 months, often because of a high index of suspicion due to a previous family history of Hurler syndrome.

Phenotype pre- transplantation (Table 6.2)

Eighteen of the children had characteristic facial appearance at diagnosis, one girl was considered to have a normal appearance when diagnosed at 2 months of age. All children had corneal clouding and some evidence of dysostosis multiplex. All had

acetabular abnormalities, 18 children had vertebral anomalies most commonly involving L1/L2 or L2/L3 and including poor formation of the anterior portion of the vertebral body of L1 in 2 children. Eighteen had some hand abnormality, most commonly tapering of the metacarpals, but only 11 had tibial abnormalities at diagnosis. Sixteen children had hepatomegaly, 10 also had splenomegaly but 3 children, aged 1 week, 2 months and 5 months, did not have organomegaly at diagnosis. Four children had a normal cardiac assessment, 5 others had left ventricular hypertrophy and a further 2 had mitral valve thickening. One child had a patent ductus arteriosus (PDA), whilst no cardiac record was available for another 7 children. Eleven children had abnormal audiological assessment prior to transplant and 14 children were considered to have developmental delay. Five children were considered to have achieved appropriate developmental milestones at the time of evaluation. These children ranged in age from 1 month to seven months, suggesting that factors other than age had contributed to neurological normality.

Haematopoietic stem cell transplantation

Nineteen children underwent Bone marrow transplantation. Nine children received sibling transplants, 5 of these were from a heterozygous sibling. There were 6 haploidentical transplants from an extended family donor and 2 of these were also heterozygotes. There were 4 matched unrelated transplants (Table 6.3). The median time to transplant for the whole group was 14 months. Those receiving matched unrelated grafts were transplanted in a median time of 8 months and those who received sibling grafts 6 months. Children who did not have a sibling donor and who underwent haploidentical transplantation from an extended family donor had the longest wait, median 14 months, range 3 months to 29 months, before transplant was performed.

Eight children, 4 males and 4 females, who were transplanted between 3.5 and 23 months of age, median 7 months, experienced poor engraftment or graft failure (Table 6.3/Table 6.4). Six children underwent second transplant (Table 6.4). The child who was 23 months at the time of first transplant was not retransplanted but is still alive 7 years later. One child who experienced poor engraftment maintains enzyme levels within the low normal range despite having less than 10% donor cells. The donor in 5 of

the 6 cases was heterozygous for the gene mutation (3 siblings and 2 haploidentical family members). The grafts failed completely between 5-12 months, median 7 months and the children were retransplanted between 16 and 31 months of age, median 19 months. (Table 6.4) Three of the retransplanted children underwent conditioning using Busulphan 4 mg/kg/day for 4 days followed by cyclophosphamide 50 mg/kg/day for 4 days whilst the other 3 received busulphan 5mg/kg/day and cyclophosphamide 60 mg/kg/day each given over 4 days. Four of the children were successfully retransplanted from the same donor with minimal transplant related morbidity. Two children who were transplanted using different donors had significant morbidity; one developed veno-occlusive disease of liver which resolved and the other an interstitial pneumonitis, most likely fibrosing alveolitis, which proved fatal.

Four other children died while undergoing transplantation. One, as already mentioned, died of respiratory failure in the UK. The other 3 received transplants at OLHSC. Two developed veno-occlusive disease of liver and the third bronchiolitis obliterans.

Post transplant course. (Table 6.5)

The children have now been followed up for between 1 and 15 years, median 8 years, since transplant. Child 10, who experienced graft failure but is still alive aged 7 years, continues to have hepatosplenomegaly and her skeletal abnormalities have worsened. Her hearing, vision and neurodevelopmental parameters have also disimproved. Three children transplanted within the last 2 years experienced resolution of their organomegaly but are otherwise unchanged. The remaining children, whose transplant was more than 4 years ago, no longer have organomegaly and some show improvement in their coarse facial features. Only 2 children, transplanted at 3 and 4 months of age, have experienced some corneal clearing. One child (no 11) who received a matched unrelated transplant at 4 months of age appears to have a significant arrest of his disease. He has only experienced slight deterioration of his skeletal lesions, not requiring surgery, and neurodevelopment during a 6 year follow up. All the other children have experienced progressive worsening of their dysostosis multiplex. Seven have undergone Salters Osteotomy and 3 have also had tibial stapling with variable success (Table 6.6). Two children have experienced motor dysfunction of the lower

limbs, in one case it appeared due to narrowing of the dorsal spinal canal and in another due to progressive kyphoscoliosis because of agenesis of the anterior half of the 1st lumbar vertebra. In the latter case spinal fusion has corrected the neurological deficit. Only 2 children have required carpal tunnel release. Three of the children who have been followed up for longer than 4 years have had neurodevelopmental stabilisation, whilst the others show continued deterioration. Eight children have experienced progressive hearing loss and require hearing aids. Only one child has shown hearing improvement and another has stable hearing loss. Left ventricular hypertrophy has resolved in 4 children and improved in another but 3 others have developed mitral valve lesions.

Discussion.

All of the children described here have the W402X mutation which gives rise to severe Hurler syndrome phenotype. None of the untransplanted children are still alive, whilst the child whose transplant failed and was not retransplanted is alive at 9 years of age with normal cardiac function. The non-transplanted life expectancy for some one with severe Hurler syndrome is 5-10 years (Peters et al, 1998b), and outcome in this case suggests that BMT conditioning may slow the disease's progress.

It has been suggested that bone marrow transplantation should be performed in children before the age of 24 months to ensure the best neurodevelopment outcome. It is also suggested that children with markedly impaired neurodevelopment at diagnosis should not be transplanted (Peters et al, 1998b; Krivit et al, 1999). All but one of the children in our group were transplanted under 24 months, nevertheless the majority have showed a continued slow neurodevelopmental decline. Only one child transplanted at 4 months has had long term neurodevelopmental stabilisation. Another child transplanted at 3 months is not long enough following transplant to draw definitive conclusions and the only other child to be transplanted at 3.5 months has experienced graft failure and so cannot be evaluated. Nevertheless it seems logical that earlier transplant should influence neurodevelopment. This has also been noted by Whitley et al who found that those with normal intellectual function prior to transplant remained normal but those

with lower intellectual scores pre transplant developed significant cognitive defects (Whitley et al, 1993).

Seven children received stem cells from a heterozygous donor and six of these experienced graft failure, the seventh (no.19) died of transplant related complications. Two other children who received normal stem cells also experienced total or incomplete loss of graft but the reason for this is more obvious and the outcome better. One child who received a normal sibling graft still has sufficient donor cells to maintain low normal enzyme levels and the other, a MUD transplant, experienced graft failure because of an inadequate conditioning regimen using busulphan 2.5mg/kg/day x 4 days followed by cyclophosphamide 60mg/kg/day x 4 days. It is suggested that the earlier conditioning regimen (Busulphan 4mg/kg/day x 4 and cyclophosphamide 50mg/kg/day x 4) contributed to increased graft failure but in our group both conditioning regimens (Table 6.4) were equally represented in those who experienced graft rejection. These data suggest that donor heterozygosity has a greater role in graft rejection than other contributors and suggests that heterozygous donors should not be used if there are any other alternatives. Matched unrelated grafts have demonstrated efficacy both in terms of survival and in neurodevelopmental outcome (Peters et al, 1996). In our group there were 4 MUD transplants. One has already been discussed, one child had a treatment related death but the other 2 have survived an in the case of one the disease phenotype has improved and he has minimal bone abnormalities. This suggests that MUD transplants could be considered in preference to heterozygous sibling transplants. The time taken to find a haploidentical family donor (Table 6.3) is currently too long to be a reasonable option, especially as outcome for MUD transplant has improved using newer conditioning regimens, and should not be considered unless there is no other donor, including MUD, available. The recent evaluation of matched unrelated umbilical stem cell transplants suggests that long-term engraftment and disease improvement can be obtained even with up to 3 HLA marker mismatch (Staba SL et al 2004), and offers an interesting alternative option for children with Hurler syndrome, especially in an Irish context (*cf* chapter 8)

Four of the children who received a second transplant from the same donor did not experience any significant morbidity but the two children transplanted using a different donor had major toxicity. This suggests that 2nd transplants can be performed but using

the same donor reduces toxicity. Interestingly our 2nd transplant outcome is better than that reported by Grewal et al who noted only 50% survival in a group of 25 patients, apparently retransplanted with normal donor stem cells (Grewal et al, 2002).

The majority of children transplanted had an expected but nevertheless disappointing outcome. Most of our patients display a progressive worsening of bone disease following transplantation which is a well recognised feature; however one patient has stabilisation of bone disease suggesting that earlier intervention may improve this outcome. The finding of major chrondrocyte abnormality in the articular surface by 4 weeks of age in the knock out mouse and the step wise progression of infiltration documented suggests that transplantation should be undertaken as quickly as possible after birth (Clarke et al, 1997). In our group four patients had worsening of their thoracolumbar kyphosis whilst the others demonstrated stabilisation of spinal lesions. One other group has commented on this but found a progressive worsening of kyphosis. Interestingly the average age at transplant in that group was 19.6 months (Vellodi et al, 1997), which may be a contributing factor.

Preliminary reports suggested that BMT would improve ocular function (Summers et al, 1996) This early optimism was not sustained however and longer follow-up (6yrs) demonstrates progressive worsening of optic function. We have not documented any major deterioration in most of our group and believe that the optic disease has stabilised. Facial features have improved in all the successfully transplanted children, in our group, although none have had complete resolution of signs. This finding concurs with those of Vellodi et al (Vellodi et al, 1997). The technology is now available for rapid detection of MPS (Chamoles et al, 2001), and early introduction of enzyme replacement therapy, which may improve or stabilise function in lungs, heart and eyes, may further improve long-term outcome although randomised controlled clinical trials will be required to determine its role in future management strategies (Wraith, 2005).

Conclusions

Most studies of bone marrow transplantation for Hurler syndrome show that it improves long-term survival, facial features, organomegaly, has a variable effect on cardiac

function and upper airways disease and neurodevelopment but no effect on bone disease. Most of these papers describe children who have been transplanted at an advanced age. Many do not evaluate the effect of heterozygous donors on transplant outcome although Peters et al show improved cognitive function in those transplanted from normal donors (Peters et al, 1998b). In many ways this study confirms the findings of these pervious studies but it also raises questions about the "best" type donor. I would suggest that transplant is best undertaken as quickly as possible following birth and certainly less that 4 month of age to achieve the best outcome. It appears that donor heterozygosity contributes to poor outcome and is best avoided. An unrelated donor transplant is now technically possible and produces a good outcome. Mismatched unrelated cords could also be considered and could be rapidly available. (*cf* Chapter 8). These options should be considered in preference to a haploidentical family donor because of the delay involved in finding such a donor. The possibilities of enzyme replacement therapy particularly if commenced shortly after birth may offer a new approach to this disease, but is not a cure for the disease. Early screening of "at risk" families and intervention with either enzyme replacement and HSCT or early HSCT alone may offer a better outcome. In the future gene therapy may be the solution for this disease but only if affected children are diagnosed as early in life as possible.

Number	Eponym	Enzyme deficiency	Stored Glycosaminoglycan	Major clinical manifestations
MPS IH	Hurler	α-L-iduronidase	DS, HS	Mental retardation, corneal clouding, heart disease, dysostosis multiplex, organomegaly, Death before 10 years
MPS IS	Scheie	α-L-iduronidase	DS, HS	Normal intelligence and life span, corneal clouding, stiff joints and heart disease
MPS IH/S	Hurler-Scheie	α-L-iduronidase	DS, HS	Phenotype intermediate between Hurler and Scheie
MPS II	Hunter	Iduronate sulphate sulphatase	DS, HS	Wide spectrum of severity Mental retardation, dysostosis multiplex, organomegaly, death before 15 yrs or Normal intelligence and variability of somatic features in the milder form
MPS IIIA	SanFillippo	Heparan –N-Sulphatase	HS	Hyperactivity, mild somatic features, profound mental deterioration, death usually in the 2nd decade
MPS IIIB	SanFillippo B	α-N-Acetyl-glucosaminidase	HS	As type A
MPS IIIC	SanFillippo C	Acetyl CoA-α-glucosaminidase acetyltransferase	HS	As type A
MPS IIID	SanFillippo D	N-Acetylglucosamine 6-Sulphatase	HS	As type A
MPS IVA	Morquio A	Galactose-6-Sulphatase	KS	Short stature, odontoid hypoplasia, normal intelligence
MPS IVB	Morquio B	β-Galactosidase	KS	Wide spectrum of severity similar to Morquio A
MPS VI	Maroteaux-Lamy	N-Acetylgalactosamine-4-sulphatase	DS	Normal intelligence, dysostosis multiplex, organomegaly, corneal clouding
MPS VII	Sly	β-glucuronidase	DS, HS	Mental retardation, heart disease, corneal clouding, dysostosis multiplex, organomegaly, wide spectrum of clinical severity with foetal and neonatal forms

Table 6.1 Classification of the Mucopolysaccharidoses (From Muenzer J, 2000)

Key: DS, Dermatan Sulphate; HS, heparin Sulphate; KS, Keratan Sulphate, MPS, mucopolysaccharidosis

Number	Sex	AAP	Facies	Cornea	Th/lu skeleton	Hands	Hips	Knees	Liver	Spleen	Heart	hearing	Neurodevelopment
1	M		AN	AN	AN	AN	AN	AN	AN	AN	-	AN	Delayed
2	F	6 Mo.	AN	AN	AN	AN	AN	AN	AN	AN	LVH	AN	Delayed
3	F	3Mo	AN	AN	AN	AN	AN	AN	AN	AN	-	AN	Delayed
4	M	6Mo	AN	AN	AN	AN	AN	AN	AN	AN	-	AN	Delayed
5	F	4Mo	AN	AN	AN	AN	AN	AN	AN	N	LVH	AN	Delayed
6	F	1 Wk	AN	AN	AN	AN	AN	AN	N	N	N	N	Delayed
7	M	7Mo	AN	AN	AN	AN	AN	AN	AN	AN	-	AN	Delayed
8	M	7Mo	AN	AN	AN	AN	AN	AN	AN	N	N	AN	Delayed
9	M	7Mo	AN	AN	AN	AN	AN	AN	AN	N	-	-	N
10	F	10Mo	AN	AN	AN	AN	AN	AN	AN	AN	N	-	Delayed
11	M	5Mo	AN	AN	N	AN	AN	N	AN	N	N	N	Delayed
12	M	4Mo	AN	AN	AN	AN	AN	N	AN	AN	AN LVF	AN	Delayed
13	F	6Mo	AN	AN	AN	AN	AN	N	AN	AN	AN LVF	N	Delayed
14	F	1Mo	AN	AN	AN	AN	AN	N	AN	AN	MR	N	N
15	F	2Mo	N	AN	AN	N	AN	N	N	N	-	N	N
16	M	3Mo	AN	AN	AN	AN	AN	AN	AN	N	MR/AR	AN	Delayed
17	M	2Mo	AN	AN	AN	AN	AN	N	AN	AN	-	AN	N
18	M	3Mo	AN	AN	AN	AN	AN	N	AN	N	PDA	AN	Delayed
19	M	5Mo	AN	AN	AN	AN	AN	N	N	N	ASD/LVH	N	N

Table 6.2 Appearance pre first transplant

Key; AAP, age at presentation; Th/lu, thoracolumbar; M, male; F, female; Mo, months; W, weeks; AN, abnormal; N, normal, -, not available; LVH, left ventricular hypertrophy, AN LVF, abnormal left ventricular function; MR, mitral regurgitation; MR/AR, mitral regurgitation & aortic regurgitation; PDA, Patent Ductus Arteriosus; ASD/LVH, atrial septal defect & left ventricular hypertrophy.

Table 6.3 Outcome of 1st Bone Marrow Transplant

Type	Sibling	HIT	MUD
N	9	6	4
Time to transplant (median)	7mo	14mo	8mo
Outcome			
Graft failure	4 (44%)	3 (50%)	1 (25%)
Death	1	2 (33%)	1
Successful	4	2	2

Key: HIT, Haploidentical transplant; MUD, matched unrelated transplant; N, number; mo, months

Table 6.4 Outcome of Bone Marrow Transplantation

No.	Age at 1st Tr	Type of Tr	Outcome	Time since Tr at last review	Facies	Cornea	Th/lu skeleton	Hands	Hips	Knees	Liver	Spleen	Heart	hearing	Vision	Neurod.
1	20m	HIT	S	15yr	I	NC	W	W	W	W	R	R	R	W	W	W
2	20m	HIT	S	12yr	I	NC	NC	W	W	W	R	R	R	W	W	NC
3	6m	Het.ST	S	12yr	I	NC	NC	W	W	W	R	R	M T	W	W	W
4	14m	Het.HIT	F/2nd Tr/dead	_	-	-	-	-	-	-	-	-	-	-	-	--
5	7m	Het.ST	F & 2nd Tr	11yr	I	NC	NC	W	W	W	R	N	R			
6	8m	Het.HIT	F & 2nd Tr	10yr	I	NC	NC	W	W	W	N	N	N	N	W	W
7	14m	HIT	Poor Graft	9yr	I	NC	NC	W	W	W	R	R	NA	NC	NC	NC
8	7m	Het ST	F & 2nd Tr	8yr	I	NC	NC	W	W	W	R	N	M T	W	W	W
9	7m	ST	F & 2nd Tr	8yr	I	NC	NC	W	W	W	R	N	MR	W	NC	W
10	23m	MUD	F	7yr	NC	NC	NC	W	W	W	NC	NC	N	W	W	W
11	4m	MUD	S	6yr	I	I	W	I	NC	MW	R	N	N	N	N	MW
12	24m	ST	S	3yr	I	NC	W+	W	W	W	R	R	I	R	N	W
13	11m	HIT	Dead	-	-	-	-	-	-	-	-	-	-	-	-	-
14	5m	ST	S	4yr	I	NC	W	W	W	W	R	R	R	W	W	CVA
15	3.5m	Het.ST	F & 2nd Tr	2yr	NC	NC	NC	W	W	W	N	N	N	N	N	NC
16	7m	MUD	Dead	-	-	-	-	-	-	-	-	-	-	-	-	-
17	3m	ST	S	2yr	I	I	NC	NC	NC	NC	R	R	NA	W	N	NC
18	9m	MUD	S	1yr	NC	NC	NC	NC	NC	NC	R	N	NA	NC	NC	NC
19	7m	Het.ST	Dead	-	-	-	-	-	-	-	-	-	-	-	-	-

Key; No., number; Tr, transplant; Th, thoracic; lu, lumbar; Neurod., neurodevelopment; m, month; HIT, haploidentical transplant; ST, Sibling transplant; Het., heterozygous; MUD, matched unrelated transplant; S, successful; F, failed; yr, year; I, improved; NC, unchanged; W, worsened; MW, minimal worsening; W+, much worse; R, resolved, N, normal from presentation, NA, information not available; CVA, cerebrovascular accident; MVT, mitral valve thickening; MR, mitral regutgitation.

Table 6.5. Demographics of graft failure

Patient No.	Sex	Age 1st Tr	Type of 1st Tr	Conditioning Regimen	Time to graft failure	Age at 2nd Tr	Type of 2nd Tr	Conditioning Regimen	Tr related Problems	Outcome
4	M	14mo	Het HIT	B 4mg/kg/d x 4 days Cy 50mg/kg/d x 4 days	5m	31m	D-HIT	B 5mg/kg/d x 4 days Cy 50mg/kg/d x 4 days	Interistitial pneumonitis	Death
5	F	7mo	Het ST	B 4mg/kg/d x 4 days Cy 50mg/kg/d x 4 days	7m	20m	SD-Het ST	B 5mg/kg/d x 4 days Cy 50mg/kg/d x 4 days	None	Success
6	F	8mo	Het HIT	B 4mg/kg/d x 4 days Cy 50mg/kg/d x 4 days	7m	19m	SD-Het HIT	B 5mg/kg/d x 4 days Cy 60mg/kg/d x 4 days	None	Success
8	M	7mo	Het ST	B 5mg/kg/d x 4 days Cy 60mg/kg/d x 4 days	7m	20m	SD-Het ST	B 5mg/kg/d x 4 days Cy 60mg/kg/d x 4 days	None	Success
9	M	7mo	ST	B 5mg/kg/d x 4 days Cy 60mg/kg/d x 4 days	5m	16m	SD-ST	B 5mg/kg/d x 4 days Cy 60mg/kg/d x 4 days	None	Success
15	F	3.5mo	Het.ST	B 5mg/kg/d x 4 days Cy 60mg/kg/d x 4 days	12m	16m	D-Het ST	B 5mg/kg/d x 4 days Cy 60mg/kg/d x 4 days	VOD	Success

Key: No., number; Tr, transplant; mo, month; M, male, F, female; Het, heterozygous; HIT, Haploidentical transplant; ST, sibling transplant; SD-, same donor, D-, different donor; B, Busulphan; Cy, cyclophosphamide, Kg, Kilogram, d, day; VOD, veno-occlusive disease of liver.

Table 6.6 Number of children who have had Surgical procedures planned or undertaken.

No.	Corneal graft	Salters Osteotomy	Tibial stapling	Carpal Tunnel release	Spinal fusion
1	Planned	Y x 2	Y	-	-
2	-	Y	Y	-	-
3	Planned	Y x 2	Y x 2	Y x 2	-
5					
6					
7	-	Y x 2	-	Y x 2	-
8	-	Y x 2	-	-	-
9	-	Y x 2	-	-	-
10	-	-	-	-	-
11	-	-	-	-	-
12	-	-	-	-	Y
14	-	Y	-	-	-
15	-	Y x 2	-	-	-
17	-	-	-	-	-
18	-	-	-	-	-

Key; No., number; Y, operation was performed; x 2, operation was performed on both sides

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Chapter 7

Carbonic Anhydrase II Deficiency and the Role of Bone Marrow Transplantation

Introduction

Osteopetrosis is a rare congenital disorder of bone resorption caused by osteoclast failure (Wilson CJ et al 2000). There are several distinct phenotypic forms of the condition. A severe autosomal recessive form and a milder autosomal dominant form, some of which have been mapped to chromosome 11q12-q13 and 1p21 are most frequently found and there are also forms of intermediate severity (Horton WA et al 2000; Van Hul E et al 2002). Autosomal dominant Osteopetrosis is also known as Marble bone disease or Albers Schonberg disease and usually presents in childhood or adolescence with fractures and mild anaemia. Less frequently cranial nerve dysfunction, dental abnormalities or osteomyelitis of the mandible are the presenting features and occasionally radiographic changes are incidentally found in asymptomatic adolescents and adults (Horton WA et al 2000).

Clinical features of severe autosomal recessive Osteopetrosis.

Affected children usually present within the first year of life and frequently within the first 3 months. Most of the symptoms are caused by bony encroachment of the marrow spaces and cranial foramina. The commonest presenting complaint is visual impairment; failure to achieve visual milestones, roving eye movements and/or squints are frequently reported. Failure to thrive and recurrent infection are also common presentations and may be due to bone marrow involvement which may also cause anaemia and extramedullary haematopoiesis with an associated hepatosplenomegaly (Wilson et al 2000). It is estimated that the cumulative risk of developing visual or haematologic impairment in the first year of life is 75% and levels off after this (Gerritsen EJA et al 1994). Macrocephaly is usual and other cranial nerve lesions including auditory nerve impairment leading to deafness and facial nerve involvement have also been noted (Horton WA et al 2000). Fractures are common and are one of the classical features of Osteopetrosis. They tend to occur after moderate trauma and are rare in infancy. The long bones are most frequently affected (Wilson et al 2000). Dental

problems include failure of tooth eruption, recurrent caries and abnormal dentinogenesis. The most severely affected children die in infancy; the probability of surviving to 6 years is about 30%, and those less severely affected rarely survive beyond the 2nd decade of life, often with poor quality of life (Gerritsen EJ et al 1994; Wilson et al, 2000).

Physiology of bone Modelling/remodelling

Bone is initially formed by the deposition of mineralised tissue at developmentally determined sites and is generally preceded by a cartilage analogue, a process known as modelling. Bone lengthens by an orderly sequence of replacing cartilage by bone (endochondrial bone formation). Bone is also formed independently of cartilage, (membranous bone) particularly in flat bones such as the skull, although it is still laid down adjacent to a cartilage template (Raisz, 1999).

Remodelling of bone begins early in fetal life and once the skeleton is fully formed in young adults almost all of the metabolic activity is a remodelling process. This involves a series of highly regulated steps that depend on the interactions of mesenchymal osteoblasts and haematopoietic osteoclasts. The initial activation stage involves an interaction between the precursor cells of both lineages, leading to differentiation, migration and fusion of the multinucleated osteoclasts (Raisz, 1999). The osteoclast then attaches to the mineralised bone surface and initiates resorption by the secretion of hydrogen ions and lysosomal enzymes particularly cathepsin K, a cysteine protease, which can degrade all the components of the bone matrix including collagen at low pH (Karsenty, 2001). Carbonic anhydrase II catalyses the formation of carbonic acid from water and carbon dioxide. Carbonic acid spontaneously dissociates to bicarbonate and hydrogen ion. The hydrogen ions (protons) are transported through the cell membrane to the bone surface by a proton pump and H+-ATPase located in the ruffled border, which is an area formed by the fusion of extensive exocytic vesicles. The acidic environment generated by the osteoclast is required for dissolution of the mineral. While protons are extruded into the resorption lacuna, chloride ions are translocated to the bone site through a Chloride (Cl-) channel resulting in the net transport of hydrochloric acid. The bicarbonate remaining in the cytoplasm is eliminated from the cell via a HCO3/Cl- exchanger, replacing the Cl(-) secreted into the resorption lacuna.

Subsequently the organic material exposed after dissolution of the mineral is hydrolysed by the lysosomal enzymes (Felix et al, 1996). The attachment of osteoclasts to bone may require specific changes in the "lining cells" on the bone surface which can contract and release proteolytic enzymes to uncover the mineralised surface. Osteoclastic resorption produces irregular scalloped cavities on the trabecular bone called Howship lacunae, or cylindrical Haversian canals in cortical bone. Once the osteoclasts have completed their work of bone removal there is a reversal phase during which mononuclear cells, which may be of macrophage lineage, are seen on the bone surface. The events during this phase are poorly understood but may involve further degradation of collagen, deposition of proteoglycans to form a "cement line", and the release of growth factors to initiate the formation phase. During the formation phase the cavity created by resorption can be completely filled in by successive layers of osteoblasts, which differentiate from their mesenchymal precursors and deposit a mineralisable matrix (Raisz, 1999).

The osteoblast stem cell is pluripotent and can also differentiate into adipocytes or marrow support cells and possibly into fibroblasts, muscle cells, or cartilage cells. Once the osteoblast has differentiated and completed its cycle of matrix synthesis, it can become a flattened lining cell on the bone surface, be buried in the bone as an osteocyte or undergo programmed cell death (apoptosis). The osteocyte is critical for maintaining fluid flow through the bone (Raisz, 1999).

Systemic regulation of bone remodelling. (Raisz, 1999)

Parathyroid hormone (PTH) is a potent stimulator of bone resorption and has biphasic effects on bone formation. There is acute inhibition of collagen synthesis with high levels of PTH but prolonged intermittent administration has been shown to increase bone formation. Plasma PTH levels increase with age and this may produce an increase in bone turnover and loss of cortical bone. 1,25 Dihydroxy vitamin D is probably critical for differentiation of both osteoclasts and osteoblasts and can stimulate bone resorption and formation under some experimental conditions. Growth factor and locally derived insulin –like growth factor can stimulate bone formation and resorption. Glucocorticoids inhibit bone formation. Thyroid hormones are critical for maintaining normal bone remodelling. The most important systemic hormone is oestrogen and

oestrogen deficiency causes an increase in resorption and a decrease in formation resulting in a decrease in bone mass.

Local regulators of bone remodelling.

A large number of cytokines and growth factors which affect bone cell functions have been identified (Fig 7.1) Recently some of the proteins that are responsible for the interaction between cells of the osteoblastic and osteoclastic lineage have been identified (Raisz, 1999). Receptor activator of nuclear factor (NF-kappaB) ligand (RANKL), a member of the tumour necrosis receptor family is produced by osteoblasts, mesenchymal cells and activated T lymphocytes and is essential for osteoclast formation, fusion, activation and survival (Hofbauer et al, 2004). RANKL acts through its specific receptor RANK (Receptor activator of NF-kappaB) which is located on osteoclasts and dendritic cells. Its action is inhibited by osteoprotegerin (OPG), another member of the TNF receptor family, which acts as a soluble neutralising receptor and inhibits osteoclast formation (Kosteniuk et al 2001; Hofbauer LC et al 2001). Other growth factors , platelet derived growth factor, PTH-related protein and fibroblast growth factor, may play an important role in physiologic remodelling and an even more important role in the remodelling associated with skeletal repair.

Pathogenesis of Osteopetrosis.

Osteopetrosis is caused by a defect of the osteoclast, resulting in failure to resorb and remodel bone which leads to narrowing of the cranial nerve foramina and abnormal bone marrow cavity formation. Histologically there is persistence of the primary spongiosa characterised by cores of calcified cartilage within bone (Felix et al, 1996). The development of the marrow cavity is delayed or absent, which contributes to the distinctive radiographic appearance of homogenously dense bone. A deficiency in remodelling primary woven bone to lamellar bone which is mechanically more competent causes the increased susceptibility to fracture of osteopetrotic bone. Tooth eruption, which is also dependant on bone resorption, is delayed or absent.

Animal Models for Osteopetrosis

There are, at least, 4 mouse and rat models of Osteopetrosis and one rabbit model (Table 7.1). Stem cell defects have been noted in 4, microenvironmental abnormalities in 3 and 2 have an unidentified defect. All but 2 of the models demonstrate a decreased number of osteoclasts. Reductions in osteoclast numbers are indicative of abnormal osteoclast development whilst increases in osteoclast numbers are thought to reflect compensatory hyperplasia in response to deficient bone resorbing activity. The ruffled border which is a characteristic of active osteoclasts is absent in several types of Human Osteopetrosis (table 7.2). This may reflect an intrinsic defect of osteoclast activity or a defect of stimulatory influence from the microenvironment. Impaired bone resorption often causes hypophosphatemia and some times hypocalcaemia may also occur (Felix et al, 1996). Osteoclast abnormalities can be cured by stem cell transplantation however microenvironmental or mesenchymal defects may not be amenable to haematopoietic stem cell transplant.

The *op* mouse has a point mutation within the coding region of the M-CSF gene. Absence of M-CSF causes a developmental defect in macrophages and osteoclasts leading to impaired bone resorption and the osteopetrotic phenotype. It does not appear to exert any effect on osteoclast generation although macrophage numbers are increased suggesting that M-CSF is an essential growth factor for cells of the osteoclast lineage (Felix et al 1996). It exerts its effect via its receptor, c-*fms* (Popoff et al, 1995). Experimentally it has been shown that following a single injection of M-CSF, osteoclasts have appeared within 2 days and have survived for 25 days. However if the same experiment is performed in a serum free medium, M-CSF absence for longer than 2 hours results in osteoclast apoptosis, suggesting that other as yet unknown factors are necessary for osteoclast survival.

The *tl* rat expresses some of the traits of the *op* mouse. There are decreased numbers of osteoclasts and pleural, femoral and peritoneal macrophages are also reduced. In this case it is believed that M-CSF is biologically inactive and bone resorption can be restored by an injection of M-CSF.

The *mi* mouse has normal numbers of osteoclasts but they do not form ruffled borders. The defective gene in the *mi* mouse, called Mitf, encodes a member of the basic-helix-loop-helix-leucine zipper (bHLH-ZIP) protein family of transcription factors that is essential for osteoclast development (Hershey et al, 2004). It is thought to have a role in the process of cell fusion (Karsenty, 2001; Felix et al, 1996). It also appears increasingly likely that Mitf has a direct influence on Cathepsin K gene expression inhibiting collagen digestion (Karsenty, 2001).

The c-*src* proto-oncogene also appears to have a role in the development of the ruffled border possibly by facilitating vesicle transport to the apical membrane of the osteoclast where the ruffled border is formed and/or to the apical membrane docking proteins that facilitate the fusion and exocytosis of these vesicles (Popoff et al, 1995). The c-*fos* proto-oncogene is a major component of the AP-1 transcription factor complex which includes several *fos* and *jun*-related genes. It is proposed that c-*fos* has an important role in signal transduction, cell proliferation and differentiation and absence of c-*fos* blocks osteoclast development at the pleuripotent stem cell stage (Felix et al, 1996).

Studies have shown that the production of oxygen-derived free radicals, particularly the superoxide anion, plays an important role in the formation and activation of osteoclasts. Oxygen derived free radicals have been localised to the osteoclast-bone interface. Some children with Osteopetrosis have deficient leucocyte superoxide production (Popoff et al, 1995).

The human osteopetroses are generally less well defined. Osteoclast morphology is abnormal in the benign childhood and adult (type I and II) osteopetroses and the ruffled borders are not seen (Table 7.2). In Craniometaphyseal dysplasia the defect is caused by a defective osteoclast proton pump (Felix et al, 1996). The causes of Malignant Osteopetrosis are less well defined. Some cases have reduced osteoclast formation but the majority have normal or increased numbers. Recently it has been suggested that, at least, some cases of AROP are due to functional abnormalities within the osteoclast. Mutations in gene encoding the a3 subunit of the vacuolar ATPase which contributes to the acidification of the extracellular compartment between to bone surface and the osteoclast have been documented in up to 50% of children with AROP (Kornak et al, 2000; Taranta et al, 2003). Others have noted mutations in the TCIRG1 gene which

encodes another 116kD subunit of the vacuolar proton pump (Frattini et al, 2000). Chloride channels have important roles in the plasma membrane and in intracellular organelles. It is believed that CIC-7 provides the chloride conductance required for efficient functioning of the H(+)-ATPase proton pump in the osteoclast ruffled membrane (Kornak et al, 2001a). It is known that mice deficient in CIC-7 Cl(-) channels have severe osteopetrosis and retinal degeneration. In humans, mutations in the genes encoding the CICN7 chloride channel have been found in Autosomal dominant Osteopetrosis, severe autosomal recessive Osteopetrosis and in intermediate forms (Kornak et al, 2001b); Frattini et al, 2003).

The role of Carbonic Anhydrase in bone remodelling:

At least eleven enzymatically active carbonic anhydrases (CA) have been recognised (referred to as CA I, II, III, IV, VA, VB, VI, VII, IX, XII, XIV) (Sly et al 2001). Several additional CA related proteins that have complete CA domains but lack enzymatic activity because of amino acid substitutions in the active site have also been recognized (Sly et al, 1995). These enzymes have important physiological functions in the body to accelerate the association of CO_2 and H_2O to form H_2CO_3 which dissociates to H^+ and HCO_3^-. Carbonic Anhydrase II (CAII) is the most catalytically active of the group with the widest tissue distribution being found in bone, kidney, brain and erythrocytes, with osteoclasts being particularly rich in CAII. Deficiency of CAII impairs the production of H^+ by the osteoclast and thus bone resorption is blocked leading to the development of osteopetrosis (Whyte, 1993). The CA gene has been defined and located on chromosome 8 (Venta et al, 1991).

Carbonic Anhydrase II deficiency:

Carbonic anhydrase II (CAII) deficiency was the first human form of Osteopetrosis to be defined at the biochemical and molecular level. It is an autosomal recessive inherited condition which gives rise to osteopetrosis, renal tubular acidosis and cerebral calcification (Sly et al, 1983). Short stature, fractures, cranial nerve compression and developmental delay are variable findings (Whyte, 1993). Severe mental retardation

occurs in the Arabic (Fathallah et al, 1994) and Japanese (Soda et al, 1996) populations but normal or only mildly abnormal development has been reported in some American kindreds. (Roth et al, 1992) There are twelve described mutations of the CAII gene. Five involve exon 2, one intron 5, and one intron 6; one is an A-C transversion at codon 92; and another a single base-pair deletion (818delG) at codon 251. However, 3 mutations (His 107Tyr, 297+1g-a, 744delA) account for >90% of the reported patients with CAII deficiency (Hu et al, 1997).

Management Strategies for Osteopetrosis

Supportive Therapy

Medical treatments of osteopetrosis have attempted to improve haematological function, reduce the osteosclerotic condition and/or improve immune function but have, at most, ameliorated the disease temporarily (Key et al, 1993; Gerritsen et al, 1994). Prednisolone has improved haematological function in some patients but has not resulted in a reduction in bone mass. Calcium deficient diets have reportedly limited further sclerosis in some patients. High dose calcitriol has resulted in clinical improvement in some patients (Key et al, 1993) but not in others (Van Lie et al 1993). The administration of interferon 1b increased superoxide generation and increased osteoclast bone resorption in some individuals with Osteopetrosis but again the results are not consistent or durable (Key et al. 1995; Madyastha et al, 2000).

Other treatments including optic nerve decompression remain controversial but may be of benefit if there is residual vision and no evidence of retinal disease (Hwang et al, 2000).

Bone Marrow Transplantation

Bone marrow transplantation, by replacing the deficient stem cell (osteoclast), is the definitive treatment for Autosomal recessive Osteopetrosis (Gerritsen et al, 1994). Successful bone marrow transplantation from a genotypically HLA-identical donor

gives an actuarial 5 year disease free survival rate of 73% but any degree of mismatching reduces the chances of engraftment and increases the BMT related problems e.g. sepsis, bleeding and interstitial pneumonia. A phenotype HLA-identical or one HLA antigen mismatch from a related donor reduces survival to 43% and a graft from a matched unrelated donor has a 5 year disease free survival of 40%. Those receiving stem cells from a HLA-haplotype mismatched but related donor has a survival of 24% (Driessen et al, 2003; Fasth et al, 1999). It is important to expedite bone marrow transplants for those with Osteopetrosis in order to preserve vision and hearing, and recently the role of haploidentical transplants has been reviewed again. It is suggested that 70% engraftment can be achieved using T-cell depleted GSCF-mobilised haploidentical stem cells (Schulz et al, 2002).

The role of BMT in the treatment of CAII deficiency is less clear, perhaps because some kindreds have a mild phenotype or the diagnosis may be delayed and the patient may have irreparable neurological damage. CAII deficiency may also be mistaken for osteopetrosis with primary neurodegeneration which has a very poor prognosis, even with transplantation (Abinun et al, 1999). It has been postulated that bone marrow transplantation is unlikely to correct RTA, (Whyte et al, 1988) but its effects on the other parameters such as developmental delay and cerebral calcification are unknown. The long-term sequelae of symptomatically treated CAII deficiency are also unclear. A recent 20 year review of 35 Saudi children with CAII deficiency demonstrated disease variability; some children had a relatively mild course whilst others developed blindness and bone marrow failure (Awad et al, 2002). Another two patients with CAII deficiency have developed restrictive lung disease and a third developed sleep apnoea at 34 years of age possibly related to uncorrected osteopetrosis (Whyte, 1993).

A kindred with CAII deficiency exists within the Traveller population. There are 6 individuals with homozygous CAII deficiency and at least 7 with heterozygous deficiency. Four children were diagnosed shortly after birth and two subsequently underwent allogeneic BMT within the first 14 months of life. Two other homozygous children were diagnosed later in life and were not considered suitable candidates for BMT. A novel mutation in exon 6 has been characterised in this kindred. I have analysed the natural history of CAII deficiency in this Kindred and the role of specific therapeutic interventions in children with this mutation.

Patients, Materials and Methods

Case Histories

Two children aged 9 and 4 months (child A and C) with osteopetrosis from two related Traveller families (family 1 and 2) were referred as possible bone marrow transplant (BMT) candidates. Subsequently their older siblings, aged 2 years and 11 months and 7 years and 8 months (child B and D), were also referred for evaluation. There was a history of consanguinity within both families. Children A and B were from family 1 and C and D from family 2. Subsequently a 3rd child (Child E), aged 2 months, born to family 2 was noted to have failure to thrive and found to be affected. A third family also with a history of consanguinity and probably related to the first had a male infant (Child F), aged 5 months, who was diagnosed with Osteopetrosis following an infective episode.

Initial assessment of the children included full blood count, renal, liver and bone electrolyte profile and acid/base estimation. Growth was assessed using Tanner charts and development measured by formal developmental scores. Audiology and ophthalmic reviews were undertaken and included measurement of electroretinogram (ERG), visual evoked potentials (VER) and brain stem evoked responses (BSER). Computerized Tomogram (CT) scan of brain was performed (Cotter et al, 2005). The association of osteopetrosis and RTA was noted and CAII levels were measured. Subsequently A and C were referred for allogeneic BMT in an attempt to prevent long-term neurological damage. The older children, (B and D) having already sustained irreparable neurological damage, were managed symptomatically. All 4 children have had repeated evaluation to monitor disease status. The 2 most recently diagnosed children (E and F) are currently being evaluated.

Family 1 have 6 children; the oldest, child B, was born in 1990. She has osteopetrosis, cerebral calcification, hearing and visual loss, and mild renal tubular acidosis. She developed multiple fractures in the first two years of life. She is developmentally delayed by 18-24 months and has short stature, height < 3rd centile, and weight at the 10th centile. Her CAII level is 0.4 u/mgHb (normal female range 5.4-15.1 u/mgHb) (Sundaram et al, 1986). Her youngest brother, child A, was born in 1995. He was assessed at 9 months of age and had osteopetrosis and renal tubular acidosis, but no

evidence of cerebral calcification. He was developmentally delayed by 2 months and was rapidly developing visual loss in his left eye. His height was at the 25th centile. CAII level was 0.3u/mg Hb (normal male range 5.5-11.7u/mgHb) (Sundaram, 1986). Three other children including the HLA compatible donor had half-normal levels and only one child had normal levels (Table 7.3).

Family 2 have four children. Child D was born in 1994 but did not have a bone marrow donor available at that time. At initial assessment she was found to have osteopetrosis, RTA, cerebral calcification and developmental delay of 2 years. She also had short stature, with height at the 3rd centile and weight at the 8th centile. She had developed serious optic nerve damage by 5 months of age which required urgent neurosurgical intervention, but this was only partially successful and she had only residual tunnel vision and poor visual acuity. She also had hearing difficulty. Her CAII level is 0.3u/mgHb (Table 7.3). Her brother, child C, born in 1996 with osteopetrosis and RTA, was assessed at 4 months of age. He had evidence of developmental delay but no cerebral calcification. His height was at the 20th centile. He developed very rapid hearing and visual loss during the period of assessment. His CAII level was also 0.3u/mg Hb. This family's youngest child (child E), born in 2003, has developmental delay, was noted to be on the 3rd percentile for height and weight and had RTA. His CAII level was also 0.3u/mg Hb.

Family 3 have one male infant born in 2002 (Child F). He was diagnosed following hospitalisation for an infective episode. He was noted to be on the 25th centile for height and the 10th for weight, has Osteopetrosis and RTA. His CT scan of brain is normal but VER and BSER are delayed. His CAII level is 0.3u/mg Hb (Table 7.3).

All of the children (A-F) had normal bone marrow function, normal haematological values and had no evidence of hepatosplenomegaly.

CA-II Measurement. (Sundaram, 1986)

The haemolysate from a normal control contains both CAI and CAII. Carbonic Anhydrase I is present I 5-6 times greater amounts than CAII. Carbonic Anhydrase I is

much more sensitive to inhibition by iodide than CAII. In the presence of 5mM sodium iodide CAI is 95% inhibited and CAII is only 16% inhibited. With this knowledge, a measure of CAII activity can be derived.

Two hundred and forty microlitres of 125mM sodium iodide is added to 3.760 ml of phenol red. One hundred microlitres of 1:100 heparinised whole blood is added to an equal volume of the sodium iodide solution and the time required for a colour change is calculated. This is termed the catalysed reaction time (Tc). Control samples are obtained by diluting 100 µl of 1:100 whole blood with an equal volume of the phenol solution prepared with triton instead of sodium iodide. The concentration of enzyme units (EU) is calculated from the formula:

$$EU(U/mgHb) = (Tuc - Tc)/Tc \times Dilution \times 1/Hb\ conc.(mg/ml)$$

Where Tuc is the uncatalysed time and Tc, the catalysed time in seconds.

The results obtained for all the families are found in table 7.3

Mutation analysis of the CAII gene by direct sequencing of individual exons amplified by PCR.

CA-II gene analysis was performed using single strand polymorphism and direct sequencing of PCR products as previously described (Hu et al, 1997). Briefly, 0.1mcg of genomic DNA isolated from peripheral blood leukocytes was used to amplify various exons by PCR. Fifty pmoles of each primer, standard PCR buffer [50mM KCL/10mM Tris-chloride, pH 8.3/2 mM $MgCl_2$/0.01% (wt/vol) gelatin], 200µM of dNTPs, and 2.5 units of Taq DNA polymerase were used. PCR conditions for exons 2 to 7 consisted of 96°C, 2 min, 1 cycle followed by 94°C, 1 min; 55°C (exons 2, 4, 5 and 7) and 52°C (exons 3 and 6), 1 min; 72°C, 2 min for 30 cycles followed by 72°C, 7 min extensions. From 30-90ng of column purified cDNA for each exon was enough for direct sequencing. Five pmol of either forward or reverse primer, used to amplify a particular exon, was also used to sequence the same exon. Each exon was sequenced in both

directions. Direct sequencing was accomplished by automatic sequencer AP1377 (PE Biosystems, CA 94404).

All patients were found to be homozygous for a novel mutation in exon 6. Twelve bps (GCTCAAGGAACC) including nucleotide 696-707 are deleted and are replaced by 4 nucleotides (CACA) [Fig 7.2]. This del_{12}/ins_4 causes a frameshift in codon 211 leading to a stop after 13 missense amino acids. The resulting protein lacks, at the C-terminal, 36 amino acids. The mutation eliminates an SduI restriction site present in the normal sequence. Exon 6 and surrounding sequences from the normal allele when amplified using forward primer (5'CCTTCCTCCTACTCTGTC) and reverse primer (5'CAAGGTCTTCTGCCTTGACT) produced a PCR fragment of 275 bp which was cut by SduI restriction enzyme digestion to yield smaller fragments of 182 and 93bp. Using the same primers the mutant allele is 267bp and is not cut by SduI.

Bone Marrow Transplantation.

Donor selection and conditioning:

Child A underwent BMT from his histocompatible heterozygous CAII deficient (3.2u/mgHb) sister. Child C received a BMT from his class I and class II HLA identical paternal aunt who had normal CA-II levels. The children were conditioned with Busulphan 20mg/kg/day over 4 days followed by Cyclophosphamide 50mg/kg/day again given over 4 days. Low molecular weight heparin (100iu/kg, once a day subcutaneously), Acyclovir (10mg/kg, three times a day intravenously) and Co-trimoxazole (240mg, twice a day by mouth after neutrophil engraftment of $>0.5x10^9/l$) were given as prophylaxis against hepatic veno-occlusive disease, Herpes virus infections and Pneumocystis carinii infections, respectively. Cyclosporin A was given intravenously then orally at a dose producing a 12 hour trough plasma level of between 100 and 200 mcg/l for 1 year post-BMT as graft versus host disease (GVHD) prophylaxis. Child A received $6x10^8$ nucleated marrow cells/kg and child C received 4.2×10^8/kg body weight. Child A engrafted on day +12 and child C on day +18 (neutrophils $>0.5x10^9/l$).

Post-transplant complications:

Hypercalcemia and hyperphosphatemia

Child A developed hypercalcemia and hyperphosphatemia at day 15 post BMT which required treatment with diuretics, dietary manipulation, and steroids. The hypercalcemia subsided by day 30. However, the hyperphosphatemia was more problematic as child A required total parenteral nutrition, and it only normalized after day 82 (Fig 7.3). Child C developed hypercalcemia and hyperphosphatemia on day 25 post BMT, but required treatment only with dietary manipulation, fluids, and diuretics.

Delayed graft-vs.-host disease of skin/liver/gut

Both children had early evidence of acute skin GVHD which resolved using intravenous steroids (2mg/kg for 10 days). Both children developed delayed GVHD of liver and gut at 6 months post transplant. Child A had grade 3 disease which failed to respond to intravenous steroids and required horse anti-lymphocyte globulin (15mg/kg x 5 days) to achieve resolution of signs and symptoms. Child C had milder GVHD (grade 2) which responded to methylprednisolone (1g/m2x 2 days) with tapering of the dose over a 1-month period.

Poor feeding:

Neither child had well-developed suck and oropharyngeal coordination. They consistently refused to feed, requiring total parenteral nutrition, nasogastric feeding and finally percutaneous gastroenterostomy tube (PEG) feeding which continued for 1 and 3 years post transplant.

Long-term follow-up post-BMT (Table 7.4)

Child A is now 84 months post BMT and his CAII level is 3.8u/mg Hb. He has normal bone marrow function, chimerism studies confirm full donor haematopoiesis and his osteopetrosis has fully resolved. (Figs. 7.4 & 7.5). Interestingly, his RTA transiently improved post transplant as evidenced by a lack of bicarbonate requirement for the first month. However, his acidosis subsequently recurred and he now receives regular bicarbonate supplementation. His hearing measured by conventional audiology testing is normal; although brain stem evoked response measurement demonstrates minor nerve

conduction abnormalities on the right side. Visual acuity was poor and he continued to demonstrate abnormal VERs. His vision remained stable for 48 months and then his left optic disc was noted to be pale and vision on the left markedly reduced at a scheduled 6 month check. Repeat VER showed increased P2 delay on the left although ERG remained normal. He remains developmentally delayed by 24 months which may, in part, be due to prolonged hospitalization post-BMT. He receives intensive educational support. His growth has been maintained and height is at the 25th centile. Computerized tomogram (CT) scan of the brain at 3 years demonstrated evidence of calcification in the frontal lobes close to the junction of the white and grey matter for the first time. A scan undertaken 2 years later suggests that this has not progressed but some additional calcification of the basal ganglia has been noted.

Child C is 72 months post BMT and is a full donor chimera, demonstrated both by erythrocyte CAII levels and also leucocyte DNA studies. His osteopetrosis has resolved and he has no evidence by CT scan of cerebral calcification. His renal tubular acidosis persists and requires bicarbonate supplementation. Audiology is normal and vision, measured by confrontation and VERs, has stabilized. However recent ophthalmic examination, 6 years following transplantation, has shown the development of bilateral optic atrophy. He has evidence of continued marked developmental delay and when assessed at 60 months of age was functioning at the 1-year level. He has some 4-5 word sentences and poor vocalisation and although he attends school cannot read or write. His growth has also decelerated and his height is now at the 10th centile.

Long-term follow-up of Non-Transplanted group (Table 7.4)

Child B and D have osteopetrosis, RTA, and extensive non-progressive cerebral calcification. Growth is restricted and height is at the 3rd centile. Child B has optic and auditory nerve conduction defects demonstrated by abnormalities of VER and BSER, but audiological testing is within normal limits. Child D has continued visual deterioration with increasing prolongation of P2 measured by VER despite optic foramen decompression. Both children are developmentally delayed but this also appears non-progressive. Child B attends normal primary school although she requires additional educational intervention and is in a class one year behind that of her peers. It is planned that she will attend the local vocational college for secondary education in 1

year's time. Child D is more significantly delayed, and recent assessment demonstrated mild-moderate intellectual disability. She attends school but is not progressing well. Child E and F have recently been diagnosed with CAII deficiency and are currently undergoing full evaluation.

Discussion

Abnormalities of the osteoclast have been shown in animal models of osteopetrosis (Lowell et al, 1996; Felix et al, 1996; Popoff et al, 1995), but until recently the only human Osteopetrosis in which the osteoclast defect had been defined was CAII deficiency. Carbonic anhydrase II deficiency causes a defect in acidification of the bone resorbing compartment by the osteoclast, preventing bone resorption and leading to cranial nerve abnormalities. Retinal dysfunction has also been reported and it is suggested that VER and ERG evaluation are particularly important in assessment of young children with osteopetrosis (Thompson et al, 1998).

Bone marrow transplantation, which reverses osteopetrosis and restores haematopoiesis, is the treatment of choice for children with malignant autosomal recessive osteopetrosis because of the poor long-term life expectancy (Taylor et al, 1995; Fasth et al, 2003), but BMT for CAII deficiency, a condition which may have significant morbidity but an ill-defined mortality risk, has not been previously described. We have demonstrated that BMT reversed osteopetrosis in CAII deficient patients and, initially, stabilized vision and hearing. (Mc Mahon et al, 2001) The recent deterioration in vision experienced by child A and C is surprising and disappointing. There is no evidence of graft failure, and Magnetic Resonance Imaging (MRI) has not demonstrated bony encroachment, but VERs are abnormal suggesting that in this group primary optic nerve abnormalities play a significant role in visual loss. These abnormalities are not amenable to transplantation or indeed optic nerve decompression, which was unsuccessfully performed in child D. Transplantation improved growth potential but no effect on the renal lesion was observed. The onset of cerebral calcification was delayed, but was not prevented in child A. It is interesting to note that child A was transplanted from a heterozygote donor, whilst child C who was transplanted from a donor with normal CAII levels has

no evidence of cerebral calcification at an age when his non-transplanted sibling demonstrated extensive calcification.

All children have some evidence of developmental delay. The children of family 2 are more severely affected, even though all four children have the same genetic mutation. This type of phenotypic variability has, also, been described in the Hispanic group (Hu et al, 1994). Initially it was felt that child A and C's prolonged hospitalisation may have contributed to their developmental delay (Mc Mahon 2001), but it is now evident that both children have ongoing problems. The causes of the developmental delay associated with CAII deficiency are unclear but are not simply associated with cerebral calcification because child C has the most marked developmental delay. It has been shown experimentally that CAII deficient oligodendrocytes show delayed maturation (Cammer et al, 1998), and it is possible that abnormalities at a cellular level, which are not corrected by transplant, also contribute to the developmental delay. The failure to arrest the developmental defect parallels the previously noted failure to restore normal renal tubular acidification. This suggests that BMT for CAII deficiency is not simply a method of enzyme replacement, as is the case with BMT for lysosomal storage diseases, but a stem cell replacement therapy which replaces osteoclasts but fails to replace other stem cells involved in renal tubular function or central nervous system formation. It also appears clear from child B's much better progress that very active, intensive educational and occupational therapy is necessary to improve the group's developmental and social attainments.

Whilst allogeneic BMT is not without risk, children with osteopetrosis undergoing transplantation have added specific complications. Both children developed transient hypercalcemia as a result of osteoclast engraftment (Gerritsen et al, 1994; Rawlinson et al, 1991) which, particularly in the case of child A, required prolonged aggressive treatment. Bisphosphonates which alleviate hypercalcemia by inhibiting osteoclast function were, however, not required.

It is known that the phenotype of CAII deficiency may vary considerably. Some patients lead relatively normal lives (Whyte, 1993; Awad et al, 2002). The treatment for such patients should be symptomatic as the risks of BMT are unjustified. Others with CAII deficiency have a much more damaging phenotype and we suggested that, as with

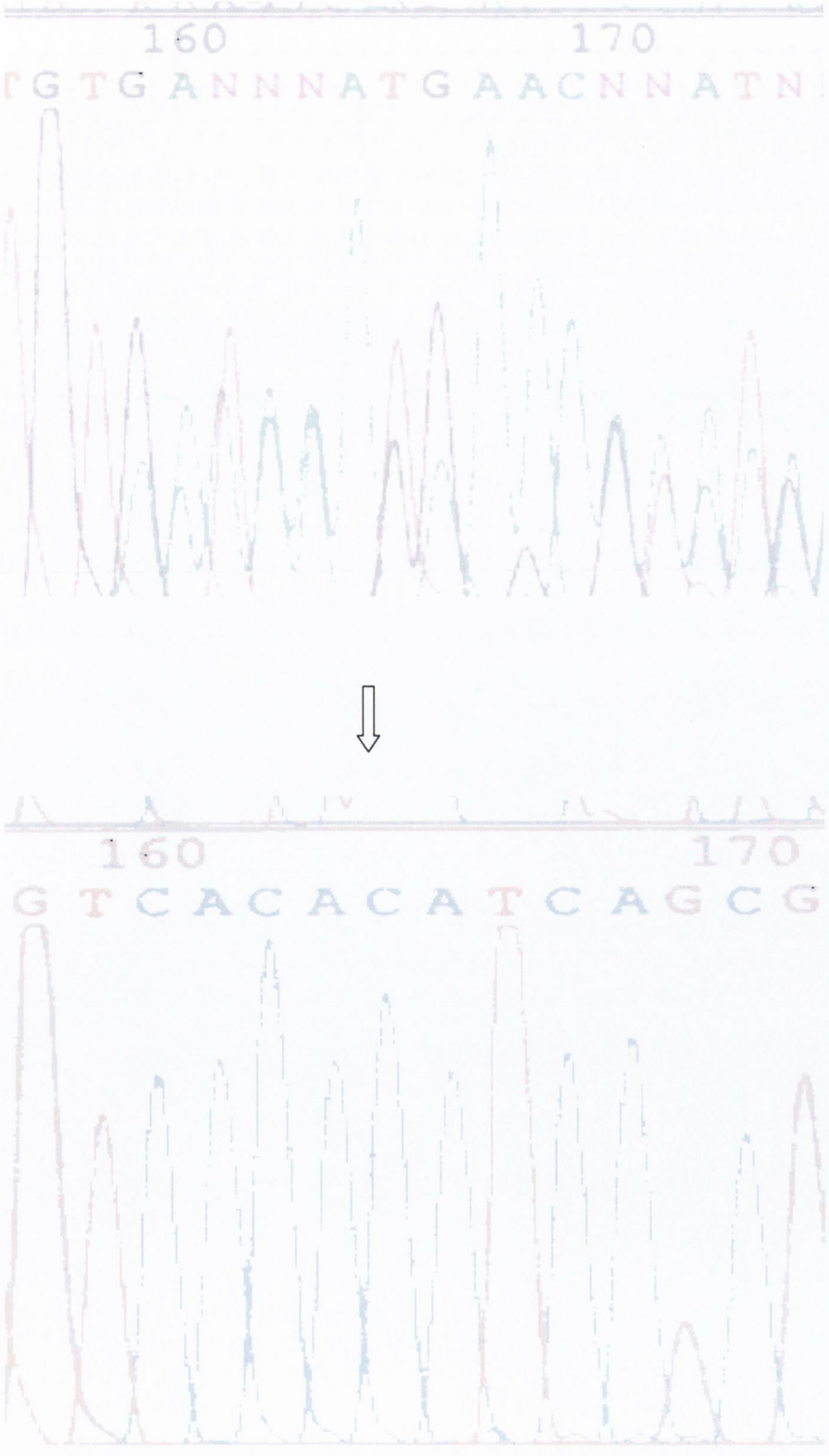
other metabolic diseases (chapter 6), early transplantation may prevent long term damage. (Mc Mahon 2001). Our ongoing analysis of this Irish group, who have a severe phenotype due to a novel mutation causing an inability to express protein, demonstrates very clearly that BMT does not alter renal or central nervous system abnormalities. The current data fails to demonstrate a clear role for BMT in the management of CAII deficiency and cannot be recommended at this time.

Fig 7.1 Local factors acting on the skeleton

Cytokines that may cause bone loss: IL-1, TNF, IL-6, ODF
Cytokines that may prevent bone loss: IL-4, IL13, IL-18, IFN, OPG, IL-1ra
Colony stimulating factors: M-CSF, GM-CSF
Prostoglandins, leukotrienes, Nitrous oxide
Growth factors: IGF, TGFβ, FGF, PDGF, PTHrP

Key: IL, interleukin; TNF, tumour necrosis factor; ODF, osteoclast differentiation factor; IFN, interferon, OPG, osteoprotegerin; M-CSF, macrocyte colony stimulating factor; GM-CSF, granulocyte-mactophage colony-stimulating factor; TGFβ, transforming growth factor-β; FGF, fibroblast growth factor; PDGF, platelet derived growth factor; PTHrP, PTH-related protein.

Fig 7.2 Gene analysis CAII
Twelve bps including nucleotide 696-707 are deleted and replaced by 4 nucleotides CACA. This causes a frameshift in codon 211 leading to a stop after 13 missense amino acids



(reproduced with the permission of Dr Gul Shah and Prof WS Sly)

Fig 7.3. Child A Ca/PO4 levels post BMT

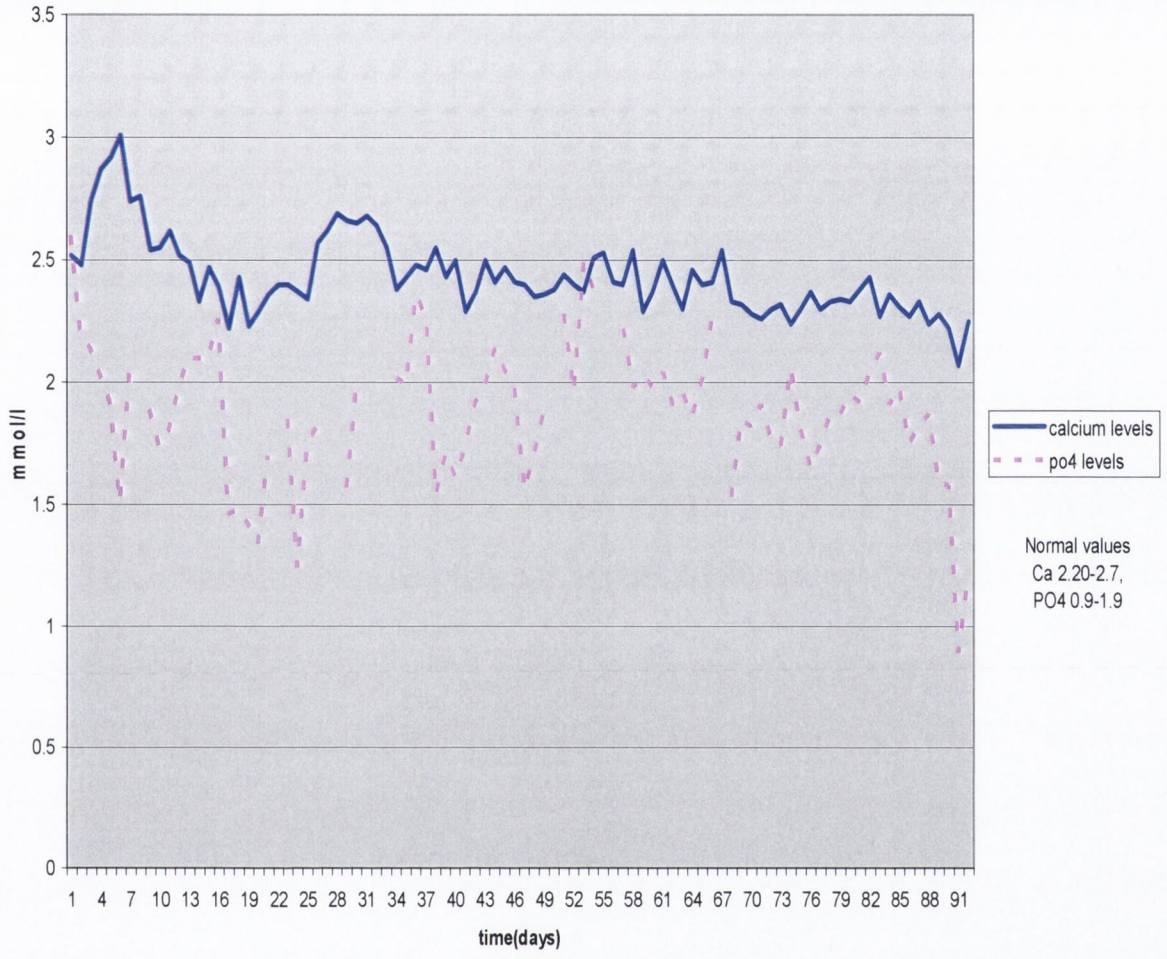


Fig 7.4 Bone Marrow appearances pre and post Transplantation

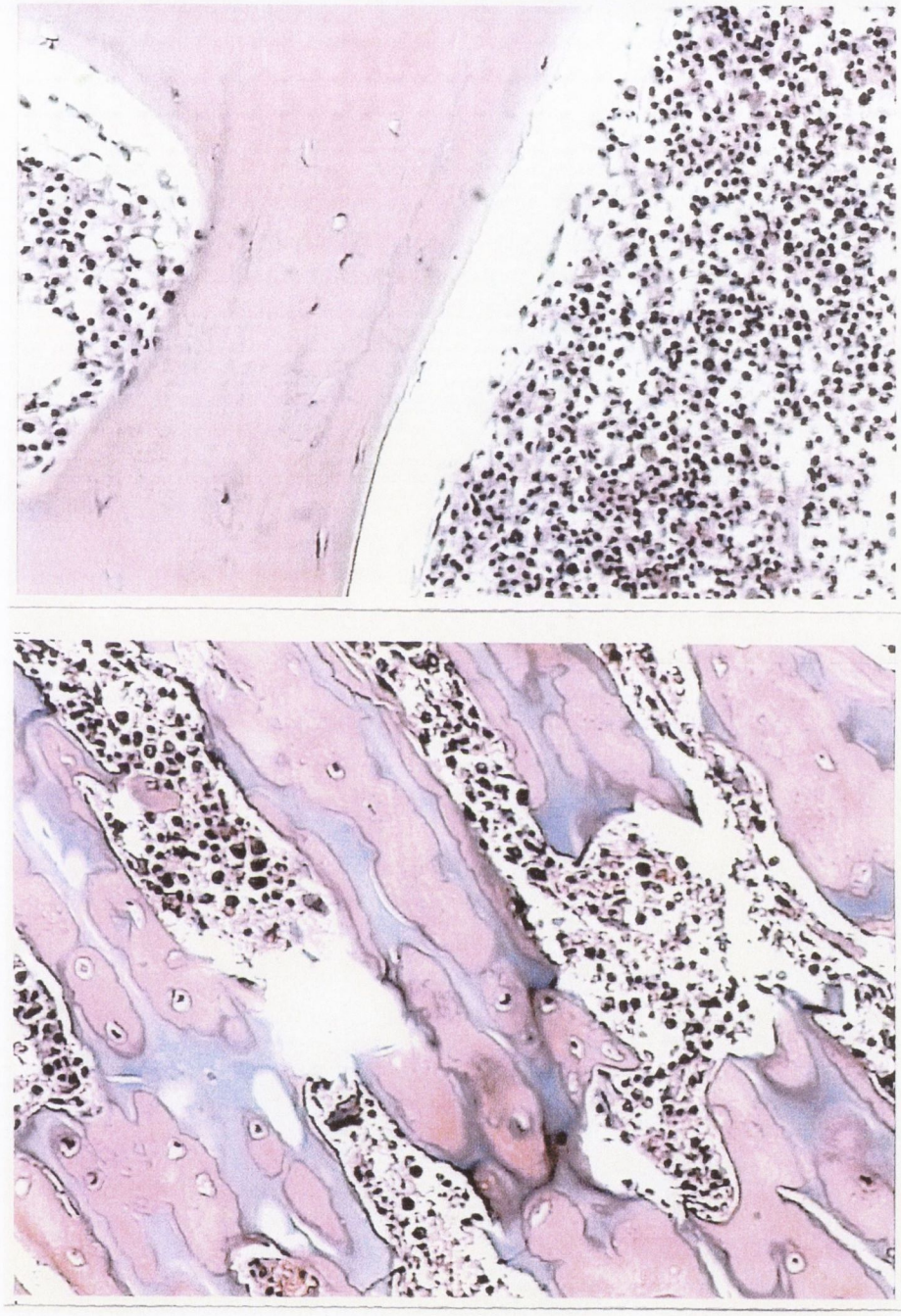


Fig. 3 Bone biopsy prior to BMT (left panel) shows bony trabeculae,to be irregular and increased in number, contain residual strips o unremodeled cartilage (blue-grey within the pink tracecular bone). Osteoclasts are not increased. The marrow cavity contain trilineage haemopoiesis but is markedly reduced. Post BMT (right panel) the bony trabeculae are normal with no redidual cartilage The marrow space is expanded by cellular trilinage haemopoiesis. Both illustrations are of haematoxylin and eosin stained sections a 140x.

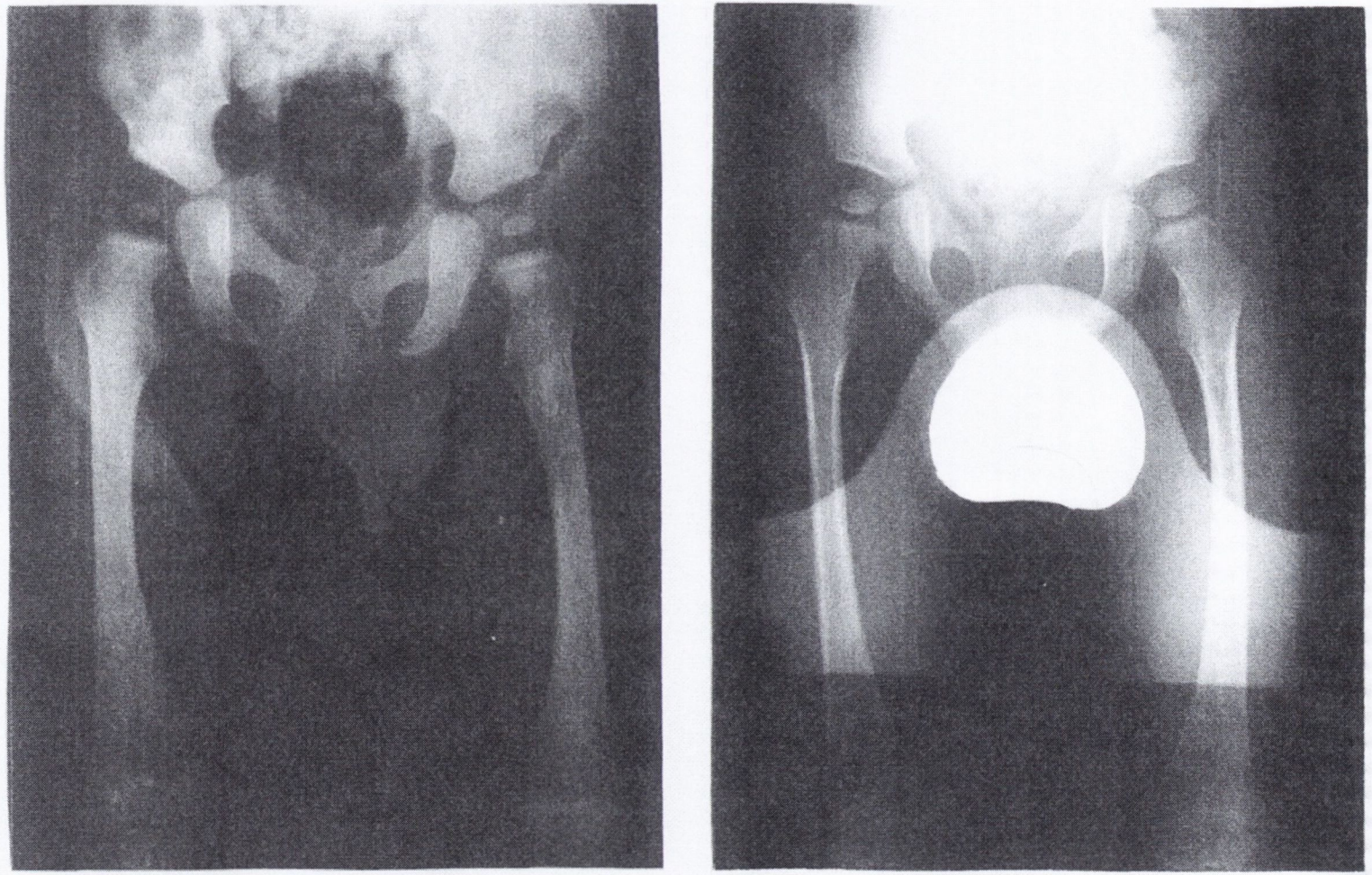


Fig. 2 X-Ray prior to BMT (left panel) shows diffuse sclerosis of the femora and ileum along with Erlen-Myer Flask deformity (Metaphyseal widening) of the distal femora. One year following BMT (right panel) shows complete resolution of sclerosis and metaphyseal widening.

Fig 7.5 Bone appearances pre and post transplantation

Table 7.1 Defects found in naturally occurring Osteopetrosis in mouse, rat and rabbit

	Intrinsic defect of	**No of osteoclasts**	**Presence of ruffled border**
Mouse			
gl (grey lethal)	Stem cells	Decreased	Yes
mi (microphthalmia)	Stem cells	Normal	No
oc (osteosclerosis)	?	Decreased	No
op (Osteopetrosis)	Microenvironment	Decreased	Yes
Rat			
*ia (*incisor absent)	Stem cells	Increased	No
tl (toothless)	Microenvironment	Decreased	Yes
op (Osteopetrosis)	Stem cells	Decreased	Yes
mib (micropthalmia)	?	Decreased	?
Rabbit			
os (Osteopetrosis)	Microenvironment	Decreased	No

Table 7.2 Abnormalities found in Human Osteopetrosis

Type	Number of Osteoclasts	Presence of ruffled border
Carbonic Anhydrase II deficiency	Normal	Yes
Craniaometaphyseal dysplasia	Normal	Yes
Type I	Decreased	No
Type II	Increased	No
"Malignant" Autosomal Recessive	Normal/Increased, seldom decreased	Yes/No

Table 7.3 Results of CA assay for Families 1, 2 and 3

Individuals	Total CA(I+II) (u/mgHb)	CAII (u/mgHb)
Family 1		
Dad	12.0	4.3
Mum	10.5	3.7
Donor	9.2	3.2
Sibling	9.4	3.7
Sibling	7.6	3.2
Sibling	15.3	6.7
Child A		0.4
Child B (Pre-BMT)		0.3
Child B (Post-BMT)	9.7	3.8
Family 2		
Dad		
Mum		3.0
Child C		0.3
Child D(pre-BMT)		0.3
Child D (Post-BMT)	18.8	8.0
Donor		8.0
Child E		0.3
Family 3		
Child F		0.3

Normal range (Sundaram et al)
Males; CAI+II 13.9-18.9, CAII 5.0-11.7
Females CAI+II 14.8-28.5 CAII 5.4-15.1

Legend: CA, carbonic anhydrase; u/mgHb, units per millegram of Haemoglobin; BMT, bone marrow transplant.

Table 7.4 Patient details pre/post BMT (* children post BMT)

Name	Sex	Age at assessment	CAII level u/mg Hb	RTA	OP	Growth	Cerebral Ca++	Eye assessment	Hearing	Development/ performance age
Initial evaluation										
Child A	M	9mo	0.3	Y	Y	Length 25th centile Wt 3rd centile	N	VER reduced L>R Optic atrophy	BSER mildly abnormal	7mo (GMDS)
Child B	F	92mo	0.4	Y	Y	Hgt 3rd centile Wt 10th centile	Y	Optic atrophy ERG abnormal VER P2 130ms	BSER abnormal L side	5yr 9mo (WISCIII)
Child C	M	3mo	0.3	Y	Y	Hgt 20th centile Hgt 15th centile	N	Optic atrophy ERG Normal VER P2 reduced latency 147ms (rapid deterioration)	BSER abnormal Low voltage waves 2,3,5 only (rapid deterioration)	6 weeks (GMDS)
Child D	F	35mo	0.3	Y	Y	Hgt 3rd centile Wt 8th centile	Diffuse parenchymal calcification	(Previous optic foramen decompression at 5 mo) ERG abnormal VER P2 116ms	BSER reduced wave 4 L side	Global delay 12 months (GMDS)
Child E	M	4mo	0.3	Y	Y	Hgt 3rd centile Wt 3rd centile	N	Pending	Pending	Pending
Child F	M	7mo	0.3	Y	Y	Hgt 25th centile Wt 10th centile	N	Optic atrophy VER P2 209ms	BSER mildly abnormal	Pending
Most recent evaluation										
Child A*	M	84mo	3.8	Y	N	Hgt 25th centile Wt 75th centile	Y Calcification in frontal lobes and basal ganglia	VER Prolonged latency P2 147 ms Normal ERG Visual acuity approx. 3/12 R side and movement only L side Optic atrophy; L>Rt	BSER L side –normal; R side –absent wave IV Audiology normal	Global delay 27mo
Child B	F	156mo	0.4	N	Y	Hgt 3rd centile Wt 10th centile	Y Both cerebellar hemispheres, cerebellar vermis, basal ganglia bilaterally, frontal lobes	Horizontal nystagmus Vision 6/36 Optic atrophy Normal ERG Abnormal VER; P2 130ms	Audiology Normal BSER abnormal bilaterally	WISCIII Verbal IQ 72 Performance IQ 70 6yr 8mo
Child C*	M	72mo	8.0	Y	N	Hgt 10th centile Wt 25th centile	N	Bilateral disc atrophy Rt>L Vision 6/9 ERG Normal VER Prolonged latency 138ms	Audiology normal Tympanometry normal L ear, flat right ear	Global delay 1yr (Bayley scales of infant development))
Child D	F	108mo	0.3	Y	Y	Hgt 3rd centile Wt 10th centile	Y	Optic atrophy Vision light perception only VER Prolonged latency P2 157ms	BSER; abnormal wave 4 L side	4yrs

Key: CAII-carbonic anhydrase II, U-Unit, Hb-Haemoglobin, Mg-milligram, RTA-renal tubular acidosis, OP-osteopetrosis, Ca++-calcification, Mo-month, yr-year, M-male, F-female, Y-yes, N-no, HGT-height, Wt-weight, L-left, Rt-right, VER-visual evoked responses, BSER-brainstem evoked responses, ERG-electroretinogram, GMDS-Griffiths mental development scale, MS-millisecond, IQ-intelligence quotient, > greater than, approx.-approximately.

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Chapter 8

HLA typing in the Traveller population and the availability of Haematopoietic Stem Cell Donors.

Introduction

In humans many of the genes associated with tissue graft rejection and immune response are located in a region known as the Major Histocompatibility Complex (MHC) on the short arm of chromosome 6. The polymorphic cell surface glycoproteins determined by these genes were originally identified as human leucocyte antigens and the MHC is also known as the HLA region.

The function of the MHC molecule.

One of the roles of the Major Histocompatibility Complex (MHC) in the immune system is to present peptides to T cells in order to initiate an antigen specific immune response. The antigen receptors of T cells are unable to recognise antigen directly; they can only recognise foreign antigens in the form of short segments of peptide bound to MHC molecules (Eren et al, 2000).

Two distinct but structurally similar classes of MHC molecules, class 1 and class II, are present on the surface of cells. The MHC class I molecule, synthesised by the rough endoplasmic reticulum, consists of 2 chains, an α or heavy chain which is associated non-covalently on the cell surface with β2 microglobulin. Only the class 1 α-chain spans the membrane (Fig 1). MHC class II molecules consist of a complex of α and β chains, which span the membrane and are associated non-covalently on the cell surface (Fig 1). Both MHC molecules have a peptide-binding groove or cleft. In the case of MHC class I this is formed by the folding of the α1 and α2 domains and in class II the α1 and β1 domains fold together (Fig 1) (Travers, 2000).

The MHC class I molecule binds short peptides. These peptides are usually octamers and nonamers, although longer peptides can be bound. The MHC molecule interacts with the N- and C- termini of the peptide binding it at both ends (Matsumura et al 1992;

Travers, 2000) (Fig 2). The side chains of the peptide also bind into specific pockets distributed along the peptide–binding cleft. These interactions allow the MHC molecules to discriminate between peptides, binding some and not others (Garrett et al, 1989; Matsumura et al, 1992; Young et al, 1994; Strominger et al, 1995). Class I MHC molecules bound to antigenic peptides are recognised by CD8+ cytotoxic lymphocytes (Rammensee et al, 1993). Peptides derived from self-proteins can be presented by class I MHC molecules but are generally ignored by the immune system. Natural Killer cells (NK) possess killer cell inhibitory receptors (KIR) which recognise self-MHC class I molecules. Thus classical HLA class I molecules may trigger cytotoxicity but also protect healthy cells from NK-mediated lysis.

MHC class II molecules bind peptides derived from extracellular, secreted or membrane associated proteins that have been degraded after their endocytosis and transport to a lysosome or an acidified proteolytic compartment of B cells and macrophages. Peptide binding to MHC class II molecules differs in a number of important respects from that of MHC class I. The most obvious difference is in the length of peptides that are bound. The majority of peptides bound to class II molecules are greater that 13 amino acids in length and there is in principle, no upper limit to the length of peptide that can be bound if the segment of the peptide which interacts with the MHC II molecule is unfolded and accessible. The peptides interact with the MHC II molecules along their length instead of interactions focused on each end (Brown et al, 1993; Stern et al, 1994). As with the class I molecules, side chains of the peptide interact with specific pockets in the class II molecules. They are recognised by CD4+ T helper cells (Eren et al, 2000).

Molecular genetics of the Human Major Histocompatibility Complex

The Human major histocompatibility complex (MHC) or Human Leucocyte Antigen (HLA) region encompasses over 4Mb of DNA (~0.1% of the genome) on the short arm of chromosome 6 at 6p21.3 (Campbell et al, 1993; Campbell et al, 1997). Over 220 genes have been located in the MHC and at least 10% of these have functions related to the immune system. The MHC is traditionally divided into class I, class II and class III regions in the order, centromere-class II, Class III , class I – telomere. The Class II region contains 17 known HLA genes and pseudogenes. Contiguous to that is the class

III region which encodes several of the components of the complement system. Telomeric to the class III region is the class I region which encodes more than 18 HLA genes, class I related genes and pseudogenes. Recently a number of genes putatively involved in inflammation have been identified in the central MHC, at the telomeric end of the class III region (Greun et al, 1997).

The class I region

The class I region spans 2Mb of DNA. This region contains three main functional class I loci, HLA-A, HLA-B and HLA-C, all of which are highly polymorphic. These genes are expressed by most somatic tissues at varying levels. In addition to their roles in the presentation of antigens to cytotoxic T lymphocytes, the expression of class I molecules at the cell surface can have other important effects in immune surveillance. Lack of class I expression at the materno-foetal interface may facilitate the survival of foetal tissue as an allograft in the maternal host. HLA-C molecules also interact with class I receptors expressed on natural killer cells and regulate natural killer cell-mediated lysis of target cells (Petersdorf et al, 1997).

Non-classical class I loci include HLA-E, HLA-F and HLA-G. These are much less polymorphic than HLA-A,-B, or-C and lead to the production of class I-related molecules of restricted tissue distribution. HLA-E is retained in the endoplasmic reticulum of the cell unless it receives a peptide from the N-terminus of another class I molecule, when it is involved in instructing NK cell receptors. HLA-G is expressed on foetal trophoblast cells and is thought to play a role in the maternal tolerance of the foetus (Milner et al, 2000).

The Class II region

The class II region spans over 800kb of DNA and contains approximately one gene every 40kb. In humans it includes loci encoding HLA-DP, DQ and DR that are expressed on the surfaces of antigen presenting cells, where they present peptides to T helper cells (Milner et al, 2000).

Class III region

The 1100kb segment of DNA that separates the class I and II regions is generally termed the class III region and contains at least 70 genes. Many of the genes in this region encode proteins with immune related functions. A cluster of genes at the telomeric end of the region include those encoding the complement components C2, C4 and factor B, 2 members of the heat shock protein (hsp70) family and the cytokines TNF, Lymphotoxin α and lymphotoxin β along with at least 3 others with potential roles in inflammation (Milner et al, 2000).

Recombination, linkage disequilibrium and the generation of polymorphism in MHC genes.

Alleles whose loci are located close together tend to be inherited together on the chromosome. If alleles are further apart on a chromosome the chances of being inherited together is reduced because of recombination during meiosis. Close linkage of some genes facilitates the perpetuation of favourable combinations of alleles e.g keeping class I or class II genes together. The concordance between physical distance and the recombination distance is not as good in some regions of the MHC. This can be attributed to linkage disequilibrium i.e. the non-random association of alleles at linked loci.

Jennings carried out the first analysis of the population genetics of pairs of alleles at 2 linked loci in 1917. He derived the classical result, that the measure of the association, Δ, between alleles A and B at 2 loci, which is given by:

$$\Delta = \text{frequency(AB)-frequency (A)frequency(B)} \qquad (1)$$

declines to 0 at a rate 1-r, where r is the recombination fraction between the loci. Thus, after n generations of random mating and in the absence of selection:

$$\Delta_n = \Delta_0(1-r)^n \qquad (2)$$

This result shows that Δ, and so the association between a pair of alleles in a population, declines rapidly towards 0 from its initial value unless the recombination fraction r, is small. This, in practice for human populations, means that r is, generally, less than

0.5%. It is because many of the loci within the HLA region are within 0.5% to 1% recombination fraction distance of each other, that linkage disequilibrium between alleles at the HLA loci is such a characteristic feature of population data on the HLA system (Bodmer, 2000).

An example of linkage disequilibrium in the Caucasian population is HLA-A1 and HLA-B8 which have an individual gene frequency of 27.5% and 15.7% respectively. It would be anticipated that they would occur together with a frequency of 4.3%, but this haplotype is present on 10% of all chromosome 6. This indicates a low frequency of recombination between HLA-A and HLA-B. Similarly recombination is observed between HLA-DQ and DR but there is only weak linkage between HLA-DQ and DP. Measurement of recombination rates has been made using microsatellite probes (Cullen et al, 1997). The recombination rate within the class II region (DRBI-DPBI) was shown to be 0.74%, whilst between the class I and II regions the rate was 0.94%. This is in keeping with the expected rate of 1% recombination per Mbp of DNA per meiosis i.e. there is little evidence of linkage. However, the rate of recombination between HLA-A and HLA-B was found to be 0.31%, which is smaller than expected for a 1.4Mpb segment of DNA.

Haplotype frequencies are better discriminators between populations than single allele frequencies. The haplotype A1,B8 is characteristic of Northern European populations while A1 occurs with comparable frequency throughout the caucasoid populations of Northern Europe to those of the Indian Subcontinent (Bodmer W, 2000).

There are many factors which can lead to significant linkage disequilibrium beyond that expected due simply to the recombination fraction in the absence of selection. Selection itself can lead to linkage disequilibrium. If an allele is being strongly selected for, then closely linked alleles will be pulled into the population due to linkage disequilibrium, a phenomenon which has been called "hitch-hiking". Thus during a rapid increase in the frequency of a particular allele there is insufficient time for recombination to disrupt the association between the selected allele and the other alleles on the haplotype initially associated with the selected allele. Other factors which may influence linkage disequilibrium include; recent population admixture, inbreeding (founder effect), random drift effects and variation due to inevitably limited sizes of population samples.

The effect of population admixture is probably small and will dissipate rapidly in the absence of linkage, as predicted by Jennings equation 2 (Bodmer W, 2000).

There appear to be 3 primary mechanisms for the generation of new classical MHC gene sequences: point mutation, recombination and microrecombination with different relative rates in different gene loci. Single nucleotide differences between MHC sequences have been attributed to point mutation caused by errors during DNA replication and repair. Recombination events seem to be responsible for the generation of new MHC alleles e.g. Human HLA-A69 coding allele has one exon that is identical to HLA-A68-coding allele and the rest of the gene is identical to HLA-A2-coding allele suggesting that it arose from a large-scale recombination event (Kaufman J, 2000). Comparison of the alleles at different MHC loci suggest that for each locus, variants are generated by a different mix of mechanisms. For human class I genes, the HLA-A gene seems to evolve primarily by point mutation with an occasional large-scale recombinational event. HLA-B gene sequences show many segmental exchanges, and a higher rate of evolution producing a larger number of alleles world-wide. Human class II alleles DQA and DQB genes appear to evolve primarily by point mutation, HLA-DPA and DPB genes evolve primarily by segmental exchange and DRB by a mixture of both.

One explanation for the appearance of new alleles is the appearance of devastating diseases such as zoonoses. It is speculated that the Black Death is responsible for the high level of A2 in Northern Europeans and that the prevalence of A1, B8 despite its association with autoimmune disease must have been due to strong selection at some point. Changes in MHC gene frequencies among Dutch immigrants to Surinam in South America were attributed to strong selection by pathogens in the new environment (de Vries et al, 1979).

The History of HLA Nomenclature

Initially a series of workshops were held in an attempt to define the leucocyte antigen system using a system that depended on a variety of serological reactions. A new nomenclature was defined that gave a series of letters and numbers to the designations

provided by the various laboratories involved (Bodmer JG et al, 2000). It consisted of a simple consecutive numbering system. In 1968 serological typing was the "gold" standard and the major effort was to obtain monospecific reagents and to define them by absorption. It soon became clear that this was impossible. The 3rd report of the WHO Nomenclature committee 1972 introduced the concept of provisional definitions designated by the letter w before the number and addressed the problem of broad and narrow splits which was then epitomised by the split of A9 into A23 and A24. The 7th Workshop and its associated 5th Nomenclature report (WHO Nomenclature Committee 1978) named a DR gene for D-related, the HLA-D determinants having been established by mixed lymphocyte culture assay. The 1987 report (Bodmer et al 1989) recognised that many of the previously unitary antigens defined by serology would be split by DNA sequencing, and nomenclature to relate the DNA sequence to the predominant serological specificities was introduced, e.g. subtypes or alleles related to B27 became B*2701 and B*2702. By 1999 there were 151 HLA-A, 301 HLA-B, 83 HLA-C, 5 HLA-E, 1 HLA-F, 14 HLA-G, 2 HLA-DA, 281 HLA-DRB, 20 HLA-DQA1, 43 HLA-DQB1, 18 HLA-DPA1, 87 HLA-DPB1, 4 HLA-DMA, 6 HLA-DMB, 8 HLA-DOA and 3 HLA-DOB alleles or 1027 alleles in total. Currently there are more than 150 new HLA alleles being assigned each year (Bodmer JG et al, 2000).

Problems associated with HLA typing

Historically HLA typing meant the assignment of HLA specificities using serological typing sera. Using these methods some antigens show cross reactivity because they have epitopes that are shared between a group antigens e.g. anti-B5 sera may cross-react with B53. In order to discriminate between B5 and B53 other sera, which do not react with B53, are used. Interpretation becomes more difficult if the cell expresses a combination of these antigens.

Identifying "splits" of certain antigens can be even more difficult. Originally splits were defined by sera that failed to identify a proportion of individuals with a certain antigen. Using monospecific sera some of these splits were identified but specific sera from some splits were not available e.g. A68 and A69 splits of A28. The problem is even more marked in class II typing because of the varying number of gene products expressed at the cell surface and Class II determination is now undertaken by DNA-

based techniques (Schreuder 2000). Recently it has also become clear that serological testing for class I antigens fails to identify mismatches which are detected using DNA based methodology (Scott et al, 1998). Error rates of 22.5-25% have been identified in typing HLA-B locus molecules using exclusively serological techniques (Lorentzen et al, 1997; Bozon et al, 1997). It is, now, suggested that it is inappropriate to recognise a serological specificity unless it has been defined by a DNA sequence (Bodmer J, 2000). However a combination of serological and DNA techniques is necessary to define the existence of certain "null" alleles. Discrimination between expressed and null alleles is particularly important for proposed donors and recipients of stem cell transplantation as an undetected mismatch due to the null allele may cause GVHD (Dunne et al, 2002).

The role of HLA in graft problems associated with Organ and Haematopoietic Stem Cell transplantation.

Solid organ transplant including renal, liver, cardiac and pancreas and haematopoietic stem cell transplantation including bone marrow, cord blood or peripheral stem cells are now common-place. Some of the major problems associated with these transplants are graft rejection or graft versus host disease (GVHD). The mechanisms governing these phenomena can be related to the MHC or HLA antigen system.

(1) Rejection. (Baker, 2000)

Hyperacute rejection

This occurs within 48 hours of solid organ transplant and is related to the presence of pre-formed antibodies in the recipient's serum which are specific for antigens expressed on the graft's vascular endothelial cells. Such antibodies constitute 2 main groups; low affinity IgM antibodies which are specific for the ABO blood group antigens and high affinity IgG antibodies directed against HLA class 1 and II antigens. The production of HLA IgG antibodies usually occurs as a result of previous immunisation by blood transfusion, pregnancy or failed allograft but may occur in 1% of the population without an obvious predisposing event. Both ABO and HLA antigens are present on the vascular endothelial cell and when blood flows through the graft the antibody comes in contact with its respective antigen triggering activation of coagulation, complement and

kinin cascades and leading to intravascular thrombosis, ischaemia and subsequent necrosis.

Acute rejection

This is found in 15-50% of patients receiving solid organ transplants and occurs between 5 days and 3 months after transplantation. The main afferent mechanism probably involves the migration of donor leucocytes, in particular dendritic cells, from the graft to regional lymph nodes where they are primed. The CD4+ T cell is central to this process and principal in orchestrating the effector mechanisms. In some animal models both CD4+ and CD8+ T cells can reject solid organ grafts independently, while in others CD4+ cells are the absolute requirement. In the clinical setting it is likely that both cell subtypes are involved. One property shared by all effector mechanisms is the binding of antigen receptors to HLA molecules on the cell surface. This binding may involve B lymphocytes producing anti-HLA antibodies, CD8+ cells lysing cells bearing allogeneic class I molecules or CD4+ cells recognising allogeneic class II molecules and releasing a potent cocktail of proinflammatory cytokines.

Graft failure occurs in haematopoietic stem cell transplantation for 2 main reasons; the T cells of the recipient will cause graft rejection when they are activated by the class I alloantigens of the donor and the natural killer cells of the recipient can do likewise when inhibition of their activation by class I molecules on the donor cells is absent. (Petersdorf et al, 2001) The risk of graft failure is related both to mismatching of HLA class I (HLA-A, B, and C) antigens and to the number of mismatches of HLA-A, B and C alleles (Petersdorf et al, 1997; Petersdorf et al, 1998; Petersdorf et al, 2001). Any given HLA antigen may be encoded by a family of HLA alleles with similar nucleotide sequences distinguishable only by DNA-based typing methods. Transplant donor recipient pairs with different HLA antigens always have different alleles but those with the same HLA antigens may have different alleles (allele mismatched). HLA allele mismatches are characterised by amino acid substitutions in the regions of the HLA molecule that bind peptides for presentation to T cells whereas HLA antigen mismatches are characterised by amino acid substitutions relevant to both protein binding and contact with T cells (Petersdorf et al, 2001).

Chronic rejection.

This problem is most often seen following solid organ transplantation and is the main cause of late graft loss. The aetiology of chronic rejection is multifactorial and consists of non-immune and immune mechanisms. It has been shown that the development of anti-HLA class I antibodies is associated with chronic rejection, and it is suggested that antibodies to Class II HLA antigens can also be responsible (Suciu Foca et al 1991a,b, Sundaresan et al 1998).

(2) Graft versus Host disease

This is predominantly associated with bone marrow or peripheral blood stem cell transplantation. It is caused by T cells from the donor that recognise foreign determinants on the new host and initiate an immune response (Theobald, 1995; Nash et al, 1996). Usually a 2 haplotype match in siblings means that the whole MHC regions of both chromosomes are identical including HLA-C (because of strong linkage disequilibrium with HLA-B) and HLA-DP. However because there is little linkage disequilibrium between DR/DQ and DP, DP mismatches have been reported in presumptive HLA identical siblings (HLA-A, B, DR, DQ) due to recombination events (Moreau et al, 1994). In a Volunteer matched unrelated donor transplant (MUD) there is no guarantee that the intervening untyped alleles will be matched. The degree of HLA mismatch is significantly related to both graft failure and GVHD (Davies et al, 1995; Prasad et al, 1999; Rufer et al, 1995). In one study High resolution HLA class I and class II typing has demonstrated Class I mismatching in 45% of serologically matched unrelated recipient-donor pairs and class II mismatching in 13% of pairs. This translated into a survival rate of 66% for those who were fully matched and had standard haematological risk compared with 31% in the mismatched group with similar haematological risk. Six percent of matched patients developed severe acute GVHD whereas 44% of those with mismatched grafts died of aGVHD. Thirty percent of those dying of aGVHD had only an HLA-C mismatch. One of 33 fully matched recipients but 4 of 36 mismatched recipients developed graft failure (El Kassar et al, 2001.

Cord Blood; an alternative source of stem cells for use in transplantation

Stem cells can be harvested from the umbilical cord (Cord blood stem cells) during the 4th stage of labour. Although insufficient cells are generally harvested to meet the requirements of adult stem cell transplantation, they have been used successfully in the Paediatric setting (Hows, 2001). A recent comparison of unrelated cord transplantation (UCT) and unrelated bone marrow transplantation for children with acute Leukaemia concluded that although there was delayed haematopoietic recovery and increased day 100 mortality in the UCT group; the outcome in terms of relapse was similar (Rocha V et al, 2001). Another study examined the outcome of mismatched cord transplants in children with leukaemia and concluded that it was a feasible procedure to cure children if it was conducted at a favourable phase of disease, (Ohnuma K, et al, 2001), although Eurocord and other groups have stated that the outcome of UCT becomes poorer with more than 2 HLA disparities based on serological typing for class I and high resolution typing for class II (Gluckman et al, 2001; Wagner et al, 2002). Cord blood may have some advantages from the more conventional methods of obtaining stem cells, in particular to provide a source of stem cells from a wider ethnic mix than the conventional donor panel (Proctor SJ et al, 2001). Other suggested advantages include (Green, 2000; Hows, 2001; Davy et al, 2004);

- The immediate availability of stored UCB where patients have a high probability of relapse.
- The low risk of transmissible disease.
- The apparently lower risk of acute and chronic GVHD
- The absence of donor risk
- The possible reduction in time between search and transplant.

The Irish Population

HLA frequencies in the Irish Population.

The Irish population has not been subject to major population shifts over the centuries and has remained a relatively homogeneous population until quite recently. An early

study by Reen et al demonstrated that HLA A1,B8; A2,B12; A2,B7; A3,B7; A2,BW40; A9,B7 and A2,B27 were the commonest HLA-A and –B haplotypes in the Irish population. A similar antigen population profile was noted in the population of the Outer Hebrides (Reen et al, 1980). Comparison of DR antigens between the north and south of Ireland, Edinburgh and Bristol showed that the commonest antigens in Ireland (North and South) and Edinburgh were DR4, DR3, DR2 DR7 DR6, DR1/DR5, but the frequency of DR1 and DR3 differed significantly in the Bristol population (Middleton et al, 1985). More recently the phenotypes of 1910 unrelated Irish blood donors recruited onto the Irish Bone Marrow Donor Panel were analysed using a combination of serology, DNA polymerase chain reaction with sequence specific primers and reverse hybridisation. The 5 most frequently occurring haplotypes and phenotypes are listed in table 1. The A1,B8; A3,B7 haplotype is found more frequently in the Irish population than in 9 other Northern European populations and A2,B44 is only found more frequently in the Cornish population. The combination B8,DR3 and B7,DR2 exists more commonly in the Southern Irish population than anywhere else in Northern Europe. Analysis and comparison of Irish phenotype data with those of a pooled U.K. population demonstrate a high frequency of HLA-A1, B7, B8, DR2 and a reduced frequency of HLA-A9, A30, B15 and DR4 in the Irish population (Finch et al, 1997).

Selection of haemopoietic stem cell donors for transplantation

Haemopoietic stem cells are used as rescue therapy following myeloablative chemotherapy for leukaemia or lymphoma, and as a method of gene transfer in some genetic disorders e.g. sickle cell disease, thalassaemia, Fanconi Anaemia and inherited metabolic disease e.g. Hurlers syndrome and Carbonic Anhydrase II deficiency. Stem cells may be obtained from bone marrow, peripheral blood or umbilical cord blood. Stem cell collection can be obtained from a variety of sources including autologous donation, an identical twin, a HLA identical sibling, a non–HLA identical sibling, a haploidentical parent, an extended family member or a HLA matched unrelated donor (MUD).

An Irish study estimated that the chance of finding a HLA identical sibling donor is 55.6% which has been in part attributed to large family size. However the same study

concluded that only 43.5% of children with autosomal recessive disorders such as Hurlers syndrome, Osteopetrosis, Severe Combined Immunodeficiency (SCID) and Fanconi Anaemia had a sibling donor. Experience in North America has found that the likelihood of finding a sibling donor for a patient with Leukaemia is 20-40% (O Riordan et al, 1992) A similar Italian study determined that likelihood of finding a sibling match was 33% but the chances of finding a unrelated HLA A, B and DR matched Italian donor was 54% (Dini et al, 2001). It is possible that some individuals from a restricted gene pool, particularly if there has been a degree of intermarriage, may also find a donor from within the extended family, although the likelihood of this is estimated at only 5-10%. Individuals, from within these populations, and without a matching family donor, may possibly obtain a phenotypic HLA match using one of the unrelated donor panels.

Irish Unrelated Bone Marrow Registry (IrBMR)

The Irish Unrelated bone Marrow Registry was established in 1989. In 2004 it had 15,693 potential donors registered, of which 15,421 were A,B and DR typed. DR typing by DNA technology was first introduced in 1994. A and B typing using DNA techniques was introduced in 2001 and currently all new donors are A, B and DR typed using DNA methodology. Three hundred and sixty search requests were received in 2002 and 338 of these were from international registries. A review of the data from 1999 showed that 90% of patient searches found an HLA-A, B, DR and DQ match. The length of time from initiation of a search to transplant is 8-12 weeks if good potential donors are found. (S Horgan, personal communication)

Bone Marrow Donors Worldwide (BMDW)

The international bone marrow registry, known as Bone Marrow Donors World wide (BMDW) maintains a world-wide data base of all the HLA phenotypes of volunteer bone marrow donors and cord blood units and coordinates their access and distribution. BMDW consists of 53 stem cell donor registries from 39 countries and 36 cord blood registries from 21 countries. In 2004 there were 9,122,656 donors and cord blood units

registered. The largest registries are the NMDP, USA (~4,000,000 donors), German registries (2,263,000), Anthony Nolan UK (351,346), Italian (304,801) and Canadian registries (217,702).

In 2002 there were 609,137 different HLA phenotypes registered, representing approximately 1% of all the theoretically possible HLA-A, B and DR split phenotypes although because of linkage disequilibrium the number of actual phenotypes will be less than those theoretically proposed. Fifty-five percent (334,926) of all registered phenotypes were unique i.e. occurring only once in the BMDW file. Most of these were typed by serological methods and it is possible that if these were typed using high resolution DNA methodologies more differences would appear at the allelic level for each HLA-A, B, C, DR and DQ locus. In 2002 one in seven bone marrow donors and cord blood units provided a new A, B, DR split phenotype. Countries such as South Africa, Argentina, China, Mexico and Spain have a high relative contribution of new phenotypes to BMDW, whilst Ireland now contributes less than 5% new phenotypes. In almost all countries, cord blood units have a much higher relative contribution of new HLA phenotypes to BMDW than the stem cell donors of the same country, indicating the success of the cord blood banks in recruiting cord blood units from ethnic minority donors. (BMDW, 2002) In 2001 only 30% of those on whom a search was undertaken actually went on to have a transplant. The reasons for this are not clear and may be due to physician choice or perhaps because of difficulties finding a suitable match. (BMDW, 2001).

The Irish Traveller population, disease and the role of stem cell transplantation

The Irish Traveller population is a minority grouping within the Irish population. It is estimated that there are 24-25,000 individuals and 4,790 Traveller family units in Ireland. (Paveepoint Traveller's centre Data) Some autosomal recessive conditions, particularly enzyme defects, are found more commonly in this group than in the general population and may be amenable to gene therapy which is currently only available by haematopoietic stem cell transplantation. Many of these diseases will cause irreversible long term damage by the slow accumulation of toxic precursor substances, often within the brain or other neurological tissue. Therefore to achieve maximum effect and to minimise long term damage these transplants must be undertaken as soon as possible

following diagnosis. The optimal donor is a fully matched genotypically identical sibling donor but because of the custom of repeated intermarriage within some family units it may be possible to find a phenotypic HLA identical donor within the extended family group. One of the major difficulties about such extended family searches is the time taken to get all of the individuals tested, a process which may take many months. Faced with such time constraints it may seem more advantageous to institute an unrelated donor search with a view to unrelated donor transplantation. However it has been shown that ethnic minority groupings within some populations may have differences in HLA phenotype to the majority population due to common shared ancestral haplotypes (Christiansen et al, 1991). This may pose difficulties in finding a suitable phenotypic match because the majority population is often the largest or only contributor to the unrelated donor panel (Armitage et al, 1999; Brown et al, 2000).

The aims of the current study.

I analysed data on a group of travellers who had HLA testing undertaken because an immediate or extended family member had an inherited disease amenable to bone marrow transplantation. I determined the HLA phenotypes and haplotypes of the group and compared the frequencies with which they occurred in the traveller group to the general Irish population (represented by the Irish Bone Marrow Registry) and the world population (represented by BMDW) to define if this Traveller group had any restricted or unique HLA phenotypes or if their haplotypes and phenotypes matched that of the general population. I calculated the likelihood of finding a HLA compatible donor from the IrBMR or the BMDW. I then used the group's HLA data to construct a "Traveller Donor Registry" and speculated if such a "Registry" would facilitate the search for an individual donor. These data may form the basis of an argument for the establishment of a directed cord bank for the traveller group.

Population, Materials and methods

Methodology of HLA testing.

Samples are collected into ACD (Acid citrate dextrose) and EDTA (Ethylene-diamine-tetra-acetic acid) tubes and transported to the national tissue typing centre for analysis within 24 hours.

Serological HLA A and B phenotyping was carried out using the National Institutes of Health complement dependant dye exclusion microlymphocytotoxicity test (Finch et al, 1997). Reagent antisera consisted of approximately 240 selected HLA-A and B allosera supplied by the United Kingdom Transplant Support Service Authority Bristol UK together with either 'extended' or 'Comprehensive' HLA Class I typing trays (GenTrak, Inc., Plymouth Meeting Pa., USA). In addition the splits of HLA A19 were confirmed using the HLA-A Low Resolution SSP typing kit (Dynal, Oslo, Norway) according to the manufacturer's instructions. Since 2001 HLA-A and –B testing has been undertaken using DNA technology.

HLA-DR typing by DNA techniques was introduced in 1994, prior to that DR typing was undertaken by serology using methodology analogous to that described for HLA-A and B typing. HLA-DRB typing was performed by the reverse hybridisation principle using Roche Amplicor HLA DRB Typing kit (Roche Diagnostic Systems, Branchburg N.J. USA) and the INNOLiPA Standard DRB kit (Innogenetics N.V., Belgium) according to the manufacturer's instructions.

Briefly DNA was extracted from fresh or frozen EDTA blood samples using proteinase K digestion and salting out (Finch et al, 1997). DNA was amplified by the polymerase chain reaction using bionylated primers specific to the second exon of the DRB1, DRB3, DRB4 and DRB5 genes. The 272-bp amplicon produced was hybridised to a set of 28 or 32 oligonucleotide probes immobilised on nylon strips. Colourimetric detection with a streptavidin-HRP/TMB or alkaline phosphatase/chromogen substrate solution revealed a unique pattern of purple lines which was analysed by computer to define the HLA type.

Population

All requests to the Irish Blood Transfusion Service for HLA typing and donor searches from 1984-2000 were analysed and data of children with inherited disease extracted. From this data a subgroup of children from the Traveller community was identified. HLA phenotypes of these children, their immediate and extended family members were defined and recorded. The nature of disease was also noted. In some cases several requests were received from the same family over an extended period of time as new members with disease were born and new extended family members tested and recorded. The partner of a family member was also tested in some cases to determine if children from that relationship could be a potential donor. This was a possibility as many of the partners were either 1st or 2^{nd} degree relatives. Initially in 1984 DR typing was not undertaken. Later DR typing was not undertaken when the initial A and B typing of extended family members showed no possibility of matching. There was no information documented about the outcome of these requests, i.e. if transplant was undertaken, and similarly no information as to whether any of the children without family donors had an unrelated donor search. My analysis is based on the likely outcome if such searches had taken place using (a) the rest of the traveller group for whom HLA types are recorded as a separate registry (Traveller Registry), (b) the established Irish Bone Marrow Registry (IrBMR) and (c) the international donor registry (BMDW).

Haplotype designation

Each family had a family tree constructed and the associated HLA phenotype was recorded for each individual family member. The most likely haplotypes were determined on the basis of these studies (Fig 8.2). The relative frequency of each haplotype was assessed by comparing it to those found in the rest of the traveller group, and data from the IrBMR. However as the Traveller Registry is an artificial entity constructed from the HLA types of many related individuals, this frequency data may be skewed. In an attempt to overcome this possible bias, a further analysis was undertaken using only the patients' haplotypes and comparing them with IrBMR data to determine if the traveller group had any unique characteristics. HLA phenotype data

was analysed in the same way to determine how frequently a phenotype occurred within the traveller group, the IrBMR and the BMDW.

The likelihood of finding a donor.

A fully HLA matched donor may be found in the immediate family group (siblings or parents) but donors may also be found in the extended family grouping because of the tradition of intermarriage within the traveller community. An analysis of all documented Traveller HLA phenotypes was performed to determine the likelihood of finding a HLA match for each one, from within the traveller group, and also from the IrBMR and the BMDW.

Methodology for Irish and Worldwide unrelated donor panel search.

The data bases of the Irish Unrelated Bone Marrow Registry (IrBMR) and the International Bone Marrow Transplant Registry (BMDW) are located at the Irish Blood Transfusion Service (IBTS), St James Hospital Campus. Permission to access these anonymised databases was obtained from Dr Emer Lawlor, Director and Mr Ciaran Dunne, Chief Medical Scientist, Histocompatiblity Laboratory. Instruction in using these databases to perform a search was given by Mr Tony Barnes, Senior Medical Scientist in Histocompatibility Laboratory IBTS. A donor search was instituted for each separate HLA type using the IrBMR and the BMDW. The number of potential matches/donors was recorded.

Performing a search using the Irish Bone marrow registry

A computerised search was performed using the broad HLA phenotype. If a HLA split type had been initially recorded it was converted to its broad type. If a 6-antigen HLA type was recorded then all 6 antigens were entered into the search engine and a 6 antigen match was requested. If only 4 antigens were recorded, a 4 antigen match was requested.

Searching the International Bone Marrow donor registry (BMDW).

A computerised search of this registry was undertaken using the precise HLA type recorded i.e. using the split rather than the broad HLA type. Some of the donor panels, which make up the BMDW, have recorded the broad type and have not undertaken the more precise typing now employed to determine the split. It is impossible to predict with certainty which way the HLA type will split and although some of the potential matches will be missed because of this approach, it gives a more precise picture of the matches available.

Only those who had a 6 antigen HLA phenotype were assessed using the BMDW. The 6 antigens were entered to the databases of the IBS and a request for a 6 antigen match was made, the results were then recorded.

The initial searches were undertaken in 2000-2001. In Feb 2002 I undertook a reanalysis of 10% of the data. I chose a proportion of those who previously had no match on the IrBMR and/or the BMDW and repeat searching produced the same result. The group who had previously recorded donors produced similar results although in some cases the number of potential donors had increased as new volunteers were added to the panels.

Results

The possibility of finding a donor

The possibility of finding a phenotypically matched donor for the patient group.

Forty six children from 33 families were documented as having a congenital disease potentially amenable to transplantation. Some of the families were interrelated (Table 8.2). One family had 3 affected children and another 10 families had 2 affected children. Twenty five children had Hurlers syndrome (HS), 12 had Fanconi anaemia (FA) and another 2 Fanconi mosaicism, 4 had carbonic anhydrase II (CAII) deficiency and one

child each had severe combined immunodeficiency (SCID), methaemoglobinaemia and haemophagocytic lymphohistiocytosis (HLH).

All of the children had samples for HLA typing taken from members of their immediate (nuclear) family. A median of 4 persons (range 1-11) were tested in each family. (Table 8.2) Twenty-five children had HLA typing performed on their nuclear family only. The other twenty one children had extended family searching which included samples from Grandparents, aunts, uncles and their partners and in some cases cousins. The number of extended family members tested ranged from 2-23 persons (median 11). The total number of family members tested ranged from 2-29 (median 11)

Twenty-nine children (63%) had a potential family donor. Twenty children had a possible histocompatible sibling donor, 4 found a matching Aunt or Uncle and 5 had a histocompatible cousin. Seventeen patients (37%) did not have a family match although seven of these did not have an extended family search undertaken. These children tended to come from smaller nuclear families (median number of members, including parents, was 3), whilst those whose median family size was five or greater were likely to have at least one immediate family donor (Table 8.3).

Twelve children who did not have a family match were found to have a HLA match on the IrBMR. A possible match for another child was found using the BMDW. The overall likelihood of finding a donor was 91% but the likelihood of finding a donor if none was available from within the family was 28%. Eight children (17%) had a match only from within the Traveller group (Table 8.4) and 5 of these had potential donors outside their own family group. Three children (6%) had no match available from any source (Table 8.5).

The possibility of finding a HLA matched donor for any member of the traveller group.

The HLA types of 417 HLA individuals were obtained from the IBTS records. These consisted of 46 patients, 56 parents, 115 siblings and the remainder either had no relationship recorded or were extended family members and partners. Two Hundred and

eleven different HLA types were determined. These types were used to form the speculative "Traveller Registry" (TR). The HLA types could be subdivided into 2 groups; Group I consisting of those on whom full A, B and DR types are recorded (129 persons) and group 2 who have A and B only or A, B, with C or only 1 DR recorded (82 individuals). Both groups had searches performed on IrBMR and TR but investigation of the BMDW was undertaken for group 1 only.

Analysis of both groups 1 and 2 (n = 211) demonstrated that the likelihood of finding a HLA match using the Traveller registry was 37% as 133 phenotypes (63%) occurred only once (Table 8.6a). The likelihood of finding a phenotypic match using the IrBMR was 60% as 85 phenotypes (40%) were not found on this registry (Table 8.6b). Fifty four phenotypes (25%) were not matched using the TR or IrBMR (Table 8.6c). Seventeen of these HLA types were incomplete and were not tested using BMDW. Thirty-seven were tested and 21 of these (57%) had a potential match using BMDW (Table 8.6c).

Analysis of group 1 only (n = 129) demonstrated similar data (Table 8.7), although because of the inclusion of the BMDW data, the likelihood of finding a donor, using all 3 registries, increased to 87% (113/129). If analysis of the TR is excluded, a phenotypic match can be found for 96 phenotypes using the 2 existing registries (IrBMR and BMDW), making the likelihood of finding a donor 74.4% (96/129). Seventeen individuals (15%) had a match exclusively from within the Traveller group (Table 8.8). Sixteen HLA phenotypes could not be matched on any of the 3 registries (Table 8.9). Analysis of the combined data from groups 1 and 2 or group 1 alone suggests that a Traveller registry provides a 10-15% additional possibility of finding a donor for individuals from within the traveller group.

HLA frequency analysis

The number of times the Traveller HLA phenotypes occur in their own group, and in other populations is measured using data from the traveller group, the IrBMR, representing the general Irish population and BMDW representing a more general world wide HLA phenotype pool. Only full phenotypes represented by group 1 are used in this comparison as the frequency with which partial HLA types (group 2) occur will be

much greater and give little additional meaningful information. The HLA type is then rated by the number of times it occurs in each of the groups and comparisons made. The patient data is analysed as a subgroup and in this case the incomplete types are included as the aim is to determine the influences large numbers of specific familial types will have on the data for the group as a whole.

The commonest HLA phenotype within the traveller group is A1,A2,B7,B14,DR1,DR7 occurring 18 times. (Table 8.10). It is also one of the commonest HLA types in the patient group occurring twice in distant cousins (table 8.11). Nine of the individuals with this HLA phenotype are from two related family groups. This HLA phenotype does not occur on the IrBMR or BMDW (8.10). The commonest patient phenotype found on IrBMR is A2,A29,B44,B44. The most frequently occurring 6 antigen patient phenotype on IrBMR is A2,A3,B7,B12,DR2,DR4 where it is found 49 times. It is the second most commonly occurring patient phenotype on BMDW, being found 215 times whereas it is found only once in the traveller group. HLA A2,A2,B7,B44,CW5,CW7,DR2,DR7 is the most frequently occurring patient phenotype found on BMDW (table 8.11).

The most frequently found traveller phenotype on the IrBMR is A1,A2,B8,B12,DR3,DR4, where it occurs 140 times. (Table 8.12) It is also found 121 times on the BMDW. It occurs only once in the traveller group. The commonest traveller HLA type on the World wide panel is A1,A29,B8,B44,DR3,DR7 which occurs over 400 times but it is found only once in the traveller group and 79 times on the IrBMR. (Table 8.13).

Seventy eight HLA phenotypes occur only once in this group of travellers (Table 8.10) and 38 of these do not occur on IrBMR. In total, Sixty five traveller HLA phenotypes are not found on the IrBMR (Table 8.12) and 62 are absent from BMDW (Table 8.13). Thirty three traveller phenotypes (25%) cannot be found on either IrBMR or BMDW (Table 8.14) and 16 of these are occur only once in the Traveller group (Table 8.9).

Haplotype analysis.

There are 126 different haplotypes found in this traveller group but only 83 have full A,B and DR types recorded. This group has been analysed to determine the frequency with which each haplotype occurs in the Traveller group and is compared with data prepared by Finch from the Irish Bone Marrow Registry in 1997. (Finch et al, 1997). The patient group has again been analysed separately because haplotypes of the larger traveller group will be influenced by the numbers of individuals tested from each family, leading to a possible overrepresentation of some types. Also in the case of the patient group some haplotypes are incomplete, lacking a DR type.

The commonest haplotype found in the whole traveller group is A3,B7,DR7 which occurs 62 times and has a haplotype frequency (HF) of 49%. (Table 8.15). It is not found in Finch's data where he records only haplotypes with a frequency of >0.5%. The haplotype A1,B8,DR3 occurs most frequently in the IrBMR (HF = 11.4%) but is 7th most common in the traveller group where it occurs 27 times (HF = 21%).

There are 62 haplotypes recorded in the patient group, 14 of which lack a DR type. The most frequently found haplotype in this group is A1,B7,DR1 which occurs 7 times (HF = 11%). It is the 3rd most common haplotype in the Traveller group as a whole but again does not feature in the top 28 haplotypes from the IrBMR. (Finch et al, 1997). The commonest Traveller haplotype, A3,B7,DR7 is ranked 3rd in the patient group whilst A1,B8,DR3, the most frequently occurring Traveller haplotype on the Irish registry is ranked 5th (HF = 3%) in the patient group (Table 8.16).

Discussion

The Irish Traveller population has a preference for intrafamily marriage believing it to strengthen the family bonds. A consequence of this practice is that over time recessive genes may become exposed and give rise to a series of diseases which are uncommon in the general population. Many of these diseases will lead to progressive functional deterioration and ultimately to death. The solution in some cases is to replace the dysfunctional or absent gene using a gene therapy. Currently the most common method

of gene transfer is haematopoietic stem cell transplantation using bone marrow or peripheral blood as a source of stem cells. Often early diagnosis and treatment may prevent the accumulation of toxic substances and allow the child to live and function normally. The degree of HLA antigen matching is critical as mismatching can lead to graft rejection or severe acute or chronic GVHD which can ultimately lead to death (Davies et al, 1995). Generally a genotypically matched sibling donor is considered to offer the best chance of cure without significant GVHD (Gustafsson A et al, 2000; Leung et al, 2001). In the case of the traveller population because of the degree of intermarriage between both the maternal and paternal families in preceding generations, an extended family member may have exactly the same HLA genotype as the proband. Indeed 19% of the donors identified for the patients in my study were aunts, uncles or cousins.

It could be argued that this study group has an overrepresentation of some families and is not a representative sample of the overall traveller population. Nevertheless it consists of HLA phenotypes from 417 different individuals or 1.7% of the traveller population and in an attempt to overcome any inherent bias I have analysed the patient population and the whole group separately. It is certainly true that this population is not in Hardy-Weinberg equilibrium and therefore calculation of population genetic statistics for this population will not be meaningful (Albert, 2000).

There are 211 different HLA phenotypes noted in this traveller population but only 129 of these had a "full" 6 antigen HLA type. This has arisen in some cases because typing was performed in 1984 – 1994 before DR typing was routinely introduced, whilst in other cases the initial HLA-A or –B typing was incompatible with the patient's type rendering further DR typing unnecessary.

The top five Traveller HLA 6-antigen phenotypes occur 56 times (43%) in the group but are found only 6 times on either the IrBMR or BMDW panels (3 on each) and the most frequently found traveller HLA type, A1,A2,B7,B14,DR1,DR7, is not represented on either panel (Table 8.10). Finch et al determined the most frequently occurring 10 HLA 6-antigen phenotypes in the Irish population in 1997. An analysis of these data and a comparison with their relative frequency in the Traveller group demonstrates striking differences (Table 8.17). The 2 most frequently occurring HLA phenotypes on

the IrBMR are each represented 5 times (3.8%) and ranked 7th in the Traveller group whilst another 2 are represented only twice and 6 of the commonest HLA phenotypes in the Irish population are not found at all in the Traveller group. Thirty three traveller 6-antigen phenotypes are not found in other populations (IrBMR or BMDW) suggesting that the traveller group has evolved at least some unique characteristics over time.

The haplotype data results are less striking. The commonest Haplotype in the traveller group is A3B7DR7, occurring 62 times, but does not feature amongst the top 28 Irish haplotypes described by Finch et al. The association with DR7 appears to be uncommon as A3B7 is represented in his group but is associated with a different DR type; DR2, DR4, DR1 and DR13 respectively. My data agrees with Finch et al that A1B8DR3 is the commonest haplotype in IrBMR but this is ranked 6th in the traveller group where it is found 27 times (Finch et al, 1997).

O Riordan et al concluded in 1992 that there was a 55.6% probability of finding a compatible sibling donor in Ireland (O Riordan et al, 1992). The results of searches for children with inherited metabolic disease, which constituted 17% of all requests demonstrated only a 38.3% probability of finding a sibling donor, whilst a group of children with acute leukaemia had a 50% probability of having a sibling donor. The explanation for the apparent discrepancy is unclear but one possible reason for this is that inherited diseases may affect one or more children from the same family. O Riordan et al demonstrate a clear relationship between family size and the likelihood of finding a HLA-identical sibling donor. She suggests that the likelihood of finding a sibling donor increases from 31.5% for those with 1 sibling to close to 100% for those with 6 or more siblings. My data show similar trends (Table 8.3), and suggest that the probability of finding a suitable donor increases with increasing family size whilst the chance of finding a donor where there are less than 2 siblings is poor. In view of this and the particular relationships within the traveller community, it may be prudent to institute an extended family search if the immediate family numbers are small. Nevertheless, even for those without a family donor, 74% of this group will have a suitable unrelated donor and with the development of DNA methodologies allowing specific allele determination the quality of donor matching is constantly improving, making unrelated donor transplantation a viable option. The majority of the group will find a donor using the IrBMR (60%), but an extra 15% would find a suitable donor if

the Travellers had a dedicated donor panel and if all 3 donor sources where available 87% of this Traveller group would find phenotypic match.

It is suggested that cord blood banks have particular benefits for those from ethnic minorities where the volunteer donor pool may largely represent the HLA phenotype of the majority community group. Currently they are of limited benefit for adult transplantation but have proved useful for the paediatric population. Cord stem cells are readily available and therefore time to transplant will be reduced (Barker et al, 2002), which would benefit our population. Mismatching appears to be less problematic as it has been noted that there is low incidence of severe GVHD in the paediatric setting even with only a 4 and 5 antigen match although recent data suggest a poor outcome if there are more that 2 HLA disparities (Yu et al, 2001; Wagner et al, 2002; Barker et al 2002). The time to engraftment of both neutrophils and platelets is longer with unrelated cord transplant (UCT) than either BMT or peripheral blood stem cell transplant (PBSCT). A recent analysis of UCT outcome suggests that the engraftment time for neutrophils is 11-41 days, median 28 and the platelet engraftment time is 19-99 days, median 56. (Davey et al, 2004). This compares unfavourably with PBSCT (neutrophils 12-19days, and platelets 11-18 days) and BMT (neutrophils 15-23 days and platelets 17-25 days) (Korbling et al, 2001). Nevertheless the ability to provide a source of stem cells within a few days of request makes this a good alternative source of stem cells where time to transplant is important (Davey et al 2004).

The question remains would this population benefit from a dedicated Cord bank? The Irish traveller population shares many HLA phenotype and haplotype similarities with the settled Irish population and 60% will find an apparently suitable match from the IrBMR. Nevertheless differences do exist. The commonest haplotype in this traveller group is quite uncommon in the general population, whilst the commonest haplotype in the general Irish population ranks only 6th in the Traveller group. Twenty-five percent of traveller HLA phenotypes, including the commonest HLA phenotype found in our study group, are not found in the Irish registry. Six of the top 10 IrBMR phenotypes are not represented in this group and the top 5 traveller phenotypes are represented only 3 times on the IrBMR.

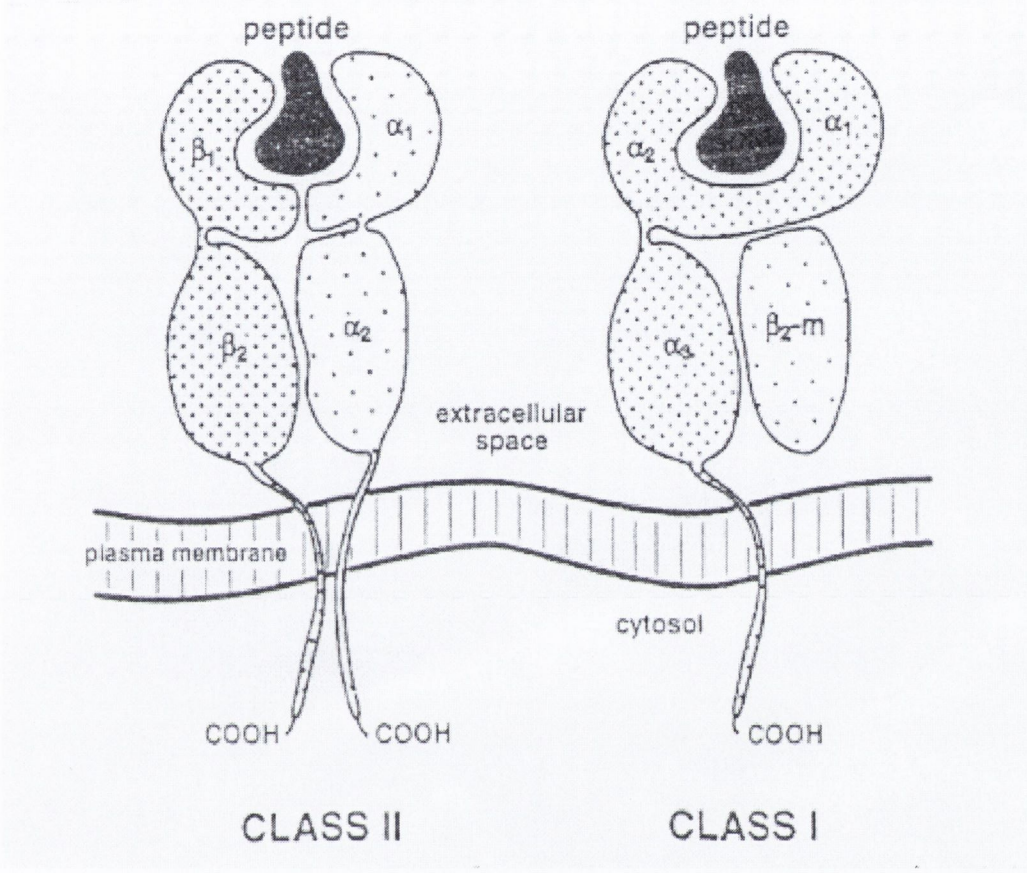
There is a 74.4% chance of finding a donor on the Irish or BMDW panels and the calculated time from search initiation to transplant is about 8-12 weeks, but there is an increased risk of severe GVHD using unrelated donor transplantation, because there may be significant allelic differences detectable only by high resolution DNA typing. The Traveller population because of its relative homogeneity may be genotypically identical even if apparently unrelated. Cord blood stem cell transplantation may reduce the risk of GVHD even further because of its low antigenic potential, allowing some HLA disparity. It will also avoid some of the antigenic risks associated with multiparous female donors and it is more likely to be CMV negative (Kollman et al, 2001). The Minnesota group calculated that cord stem cell transplantation was performed a median of 25 days sooner than bone marrow transplantation and could shorten the time from diagnosis to transplant by up to 2 months (Barker et al, 2002). This would obviously have major benefits for our group.

Conclusions

The Irish Traveller group has many characteristics that suggest its roots lie within the Irish population rather than originating as a separate group or migrating from elsewhere. It seems to have developed some unique HLA types over time but nevertheless the majority of those analysed will find a match from within their own family or within the general Irish population (IrBMR). Metabolic disease is particularly common in this group. Haematopoietic stem cell transplantation may ameliorate or cure some of these diseases e.g. Hurlers Syndrome which will cause irreparable damage if the underlying defect is not corrected quickly (*cf* Chapter 6). Other diseases found in Travellers, such as HLH, run a variable course (Chapter 5) and Fanconi Anaemia progresses slowly over a number of years (Chapter 4). Therefore only some diseases require early diagnosis and urgent transplantation. The use of cord stem cells would facilitate earlier transplant but this is not the only consideration as some time will be required to organise the transplant. Setting up and administering a dedicated cord bank as well as organising the collection and transport of the stem cells would have economic implications. It could be argued that these diseases whilst devastating for the families involved are not the major cause of death in the Traveller community, and that the money invested in such a project might be more useful if invested in the local communities to improve basic

traveller health and education. Nevertheless the concept of developing a Irish cord bank, which would include all ethnic and cultural minorities, such as the Traveller community and the recent immigrants from Africa and Asia, is an attractive one and should be considered carefully because the cord blood stem cell is, at a minimum, a useful adjunct to the currently available sources of stem cells in the paediatric population.

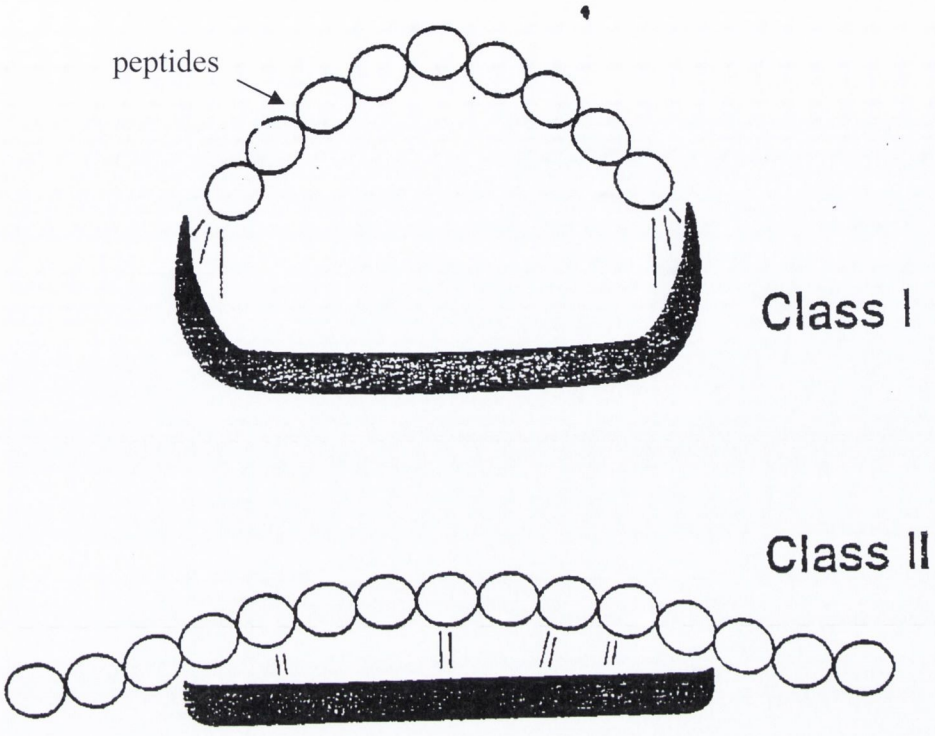
Fig 8.1 Schematic representation of the structure of MHC class 1 and Class II proteins



Key: MHC, Major Histocompatibility Complex; β_2M, beta 2 microglobulin

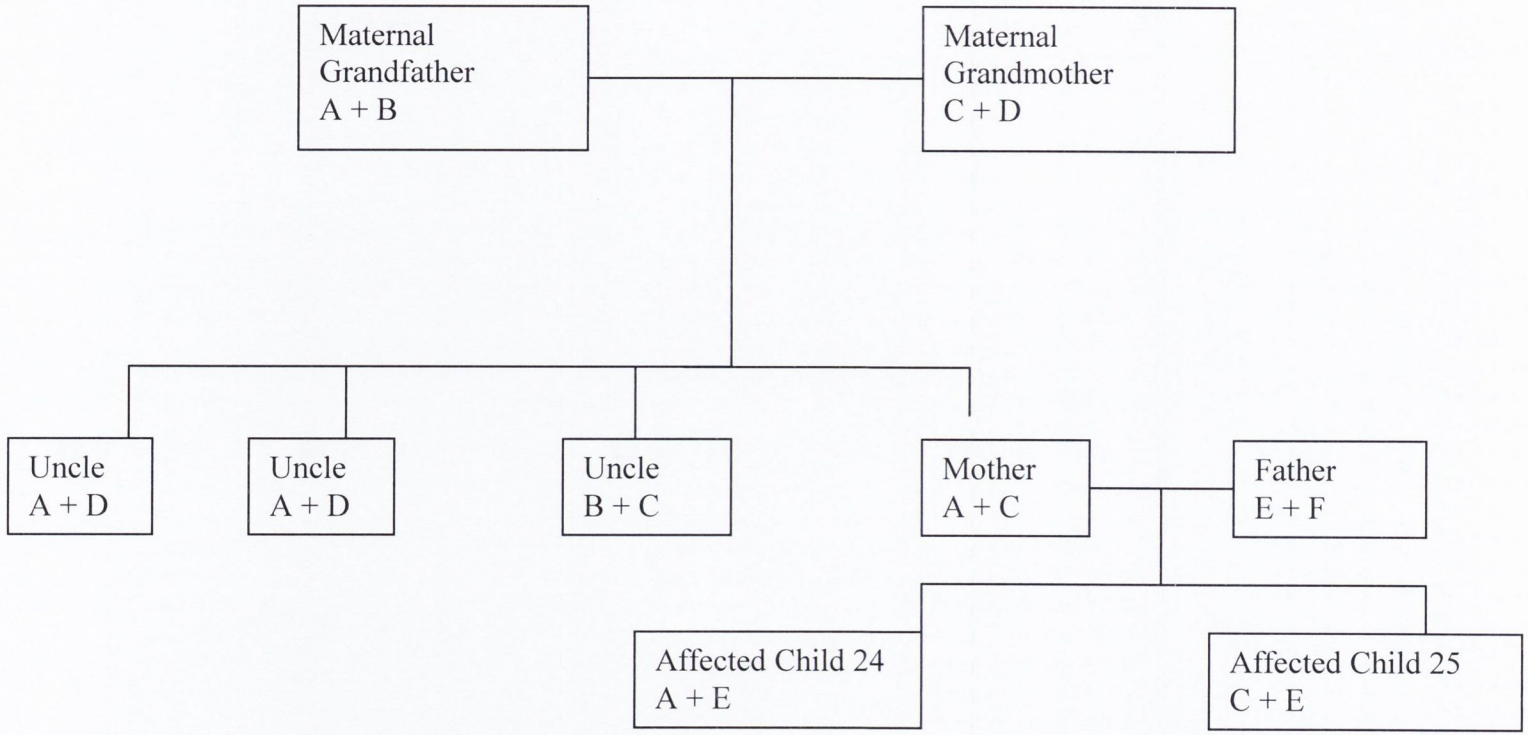
(*from Marsh et al 2000*)

Fig 8.2 Detailed analysis of peptide binding by MHC class I and II molecules



(*from Marsh et al 2000*)

Fig 8.3 Example of Haplotype Designation in a Hurlers Kindred (2 Children with Hurler syndrome No.24/25 [Table 8.3])



A = A2B14DR7
B = D = E = A1B7DR1
C = A28B14DR6
F = A1B8DR3

Table 8.1

The most frequently occurring HLA Haplotypes and Phenotypes in an Irish bone marrow Donor Panel (Finch et al 1997)

2 Antigen haplotype	**3 antigen Haplotype**	**Phenotype**
A1B8	A1B8DR3	A1A3B7B8DR15DR3
A2B44	A3B7DR2	A1A2B8B44DR3DR4
A3B7	A2B44DR4	A1-B8-DR3-
A2B7	A29B44DR7	A1A11B7B8DR15DR3
A29B44	A2B7DR2	A1A2B7B8DR15DR3

Table 8.2 Patient and family details and results of donor search

Patient No	Disease	No of immediate family members	No of extended family members	Total No of family members tested	No of matches	type of Match
1	Hurler	3	0	3	0	
2*	Hurler	4	11	15	0	
3*	Hurler	4	11	15	0	
4#	Hurler	7	0	7	3	sibs
5#	Hurler	7	0	7	0	
6	Hurler	6	23	29	0	
7^	Hurler	4	20	24	0	
8^	Hurler	4	20	24	0	
9	Hurler	3	14	17	0	
10'	Hurler	2	18	20	0	
11'	Hurler	2	18	20	1	cousin
12	Hurler	4	0	4	1	sib
13	Hurler	3	21	24	0	
14"	Hurler	0	2	2	1	cousin
15"	Hurler	2	0	2	2	sibs
16"	Hurler	6	0	6	1	sibs
17	Hurler	6	0	6	4	sibs
18	Hurler	7	13	20	1	2nd cousin
19	Hurler	9	0	9	1	sibs
20	Hurler	3	0	3	3	sibs
21	Hurler	5	0	5	2	sibs
22	Hurler	5	0	5	2	sibs
23	Hurler	4	2	6	0	
24]	Hurler	2	3	5	2	uncles
25]	Hurler	2	3	5	1	uncles
26	FA	6	18	24	2	cousins
27	FA	7	0	7	1	sibs
28	FA	3	0	3	0	
29	FA	1	17	18	1	Cousin
30	FA	8	0	8	0	
31	FA	7	0	7	1	sib
32}	FA	7	20	27	1	sib
33}	FA	7	20	27	1	sib
34+	FA	7	0	7	1	sib

Table 8.2 *continued*

Patient No	Disease	No of immediate family members	No of extended family members	Total No of family members tested	No of matches	type of Match
35+	FA	7	0	7	1	sib
36~	FA	11	0	11	3	sib
37~	FA	11	0	11	6	sib
38	SCID	1	11	12	0	
39·	CAII	6	0	6	1	sib
40·	CAII	6	0	6	3	sib
41°	CAII	2	22	24	2	Aunts
42°	CAII	2	22	24	4	Aunts/GM
43	Meth-Hb	3	0	3	0	
44	HLH	3	0	3	1	Sib
45¥	HLH/FAmosaic	3	0	3	0	
46¥	FA Mosaic	3	0	3	0	

Table 8.3 Relationship of nuclear family size to likelihood of finding a sibling donor in the Traveller group

No. of Individual patients	No. of possible sibling donors	Mean no. of nuclear family members*	Median no. nuclear family members*	Range of nuclear family size*
17	0	2.6	3	1-8
11	1	6.3	7	3-9
3	2	4.0	5	1-5
4	3	6.7	6	3-11
1	4	-	6	N/A
1	6	-	11	N/A

* including parents

Table 8.4 Details of children with HLA matchs found only within the Traveller population

Patient No.	HLA type	Disease	No. of Family matches	type of Match	Fr in T group	No. other Matches in Traveller group	Fr in IrBMR	Fr in BMDW
4	A1A2B7B14DR1DR7	H	3	sibs	18	14	0	0
14	A2A3B7B18DR2DR7	H	1	cousin	5	3	0	0
15	A2A3B7B18DR2DR7	H	2	sibs	5	2	0	0
24	A1A2B7B14DR1DR7	H	2	uncles	18	15	0	0
25	A1A28B7B14DR1DR6	H	1	uncles	2	0	0	0
33	A23A25B5B44DR4DR7	FA	1	sibs	2	0	0	0
40	A2A28B8B49DR3DR4	CAII	3	sibs	5	1	0	0
44	A3A28B7B14DR7DR13	HLH	1	Sibs	2	0	0	0

Key; No., number; Fr, frequency; T, Traveller; IrBMR, Irish Bone Marrow registry; BMDW, International Bone Marrow registries;
H, Hurler syndrome; FA, Fanconi Anaemia; CAII, Carbonic Anhydrase II deficiency; HLH, Haemophagocytic lymphohistiocytosis; Sibs, Sibling.

Table 8.5 HLA types of children without potential matches on any panel

Patient No.	HLA type	Frequency in p. group	Disease	No. of Family matches	Fr. in T group	Fr. IrBMR	Fr. BMDW
6	A3A28B14B60DR2DR6	1	HS	0	1	0	0
10	A2A28B14B53DR3DR5	1	HS	0	1	0	0
13	A1A30B8B18DR3DR15	1	HS	0	1	0	0

Key: p, patient; no., number; Fr, frequency; T, Traveller; IrBMR, Irish Bone Marrow registry; BMDW, International donor registry; HS, Hurler syndrome

Table 8.6a HLA types occurring once only in the "Traveller registry"

HLA TYPE	Frequency in Travellers	Frequency in IrBMR	Frequency BMDW
A11A28B7B53DR5	1	0	X
A11A29B27B44DR1DR7	1	1	95
A11A32B8B44	1	3	X
A11A32B8B61DR3DR15	1	0	0
A2A12B8B44	1	0	X
A1A11B7B8	1	45	X
A1A11B8B14DR3DR6	1	1	13
A1A11B8B14DR6DR6	1	0	2
A1A1B7B15DR4DR4	1	0	0
A1A1B7B62DR1DR4	1	0	5
A1A1B7B7DR1DR4	1	0	8
A1A1B7B7DR7	1	0	X
A1A1B7B8DR1DR3	1	4	4
A1A1B7B8DR1DR7	1	0	10
A1A1B7B8DR3DR4	1	4	0
A1A1B8B57DR3DR7	1	48	0
A1A1B8B62DR3DR4	1	0	0
A1A1B8B8DR3	1	226	X
A1A24B8B35DR3DR4	1	1	0
A1A28B14B15DR4	1	1	X
A1A28B14B21DR5DR6	1	0	0
A1A28B27B49	1	1	X
A1A28B8B14DR1DR13	1	1	0
A1A28B8B40DR3	1	8	X
A1A29B8B44DR3DR7	1	79	>400
A1A29B8B44DR7DR14	1	1	142
A1A2B14B35DR7DR11	1	2	0
A1A2B14B62DR4DR7	1	0	0
A1A2B7B14DR1DR1	1	0	16
A1A2B7B15	1	?	X
A1A2B7B18DR2DR4	1	1	0
A1A2B7B21DR1DR5	1	0	0
A1A2B7B40DR1DR4	1	0	5
A1A2B7B62DR1DR4	1	3	55
A1A2B7B62DR4DR4	1	3	71
A1A2B8B12DR3DR4	1	140	121
A1A2B8B14DR2DR3	1	1	0
A1A2B8B14DR3DR7	1	0	533
A1A30B8B18DR3DR15	1	0	0
A1A31B7B60DR1DR4	1	0	22
A1A3B14B18	1	2	X
A1A3B21B40DR2DR5	1	0	0
A1A3B7B14	1	11	X
A1A3B7B14DR7	1	4	X
A1A3B7B60DR1DR2	1	1	0
A1A3B7B7DR1DR6	1	0	11
A1A3B7B7DR4DR6	1	0	12
A1A3B8B27DR3DR9	1	1	0
A1A3B8B60DR3DR15	1	2	0
A1A9B5B7DR1DR5	1	1	0
A24A28B44B51DR13	1	0	X
A28A28B44B53DR5DR7	1	0	X
A28A30B13B27	1	0	X
A29A31B7B44DR1DR7	1	3	15
A2A10B5B27	1	1	X
A1A10B5B27DR1	1	1	X
A2A11B18B62	1	2	X
A2A11B44B62	1	4	X
A2A11B7B44DR1DR2	1	3	6
A2A19B40B40DR4DR4	1	0	6
A2A23B27B44DR1DR7	1	0	163
A2A24B14B35	1	1	X
A2A24B5B7C1C7	1	13	X
A2A25B18B44	1	17	X
A2A25B5B18DR4	1	2	X
A2A25B5B27DR1DR4	1	0	66

HLA TYPE (Table 8.6a)	Frequency in Travellers	Frequency in IrBMR	Frequency BMDW
A2A28B14B14DR6DR7	1	0	0
A2A28B14B44DR7	1	8	X
A2A28B14B53DR3DR5	1	0	0
A2A29B7B44	1	81	X
A2A2B13B44	1	13	X
A2A2B14B14	1	6	X
A2A2B14B14DR7DR7	1	2	0
A2A2B14B18DR1DR2	1	0	5
A2A2B14B27	1	1	X
A2A2B14B40DR4DR7	1	1	13
A2A2B14B44DR7DR11	1	3	0
A2A2B14B44DRB10701DRB111XX	1	3	49
A2A2B14B62DR4DR7	1	1	0
A2A2B44B44C5C5	1	117	X
A2A2B44B49DR4DR13	1	0	97
A2A2B7B12	1	95	X
A2A2B7B40DR2DR4	1	5	93
A2A2B7B44	1	95	X
A2A2B7B7DR2DR4	1	2	192
A2A30B13B27DR1	1	0	X
A2A32B49B61	1	4	X
A2A3B14B40	1	62	X
A2A3B14B60	1	7	X
A2A3B18B27DR1	1	0	X
A2A3B35B60DR4DR15	1	0	6
A2A3B7B12DR2DR4	1	49	215
A2A3B7B14	1	29	X
A2A3B7B14DR1	1	3	X
A2A3B7B14DR6DR7	1	1	8
A2A3B7B14DRB10701DRB10701	1	1	18
A2A3B7B14DRB10701DRB11501	1	11	90
A2A3B7B18DR2DR2	1	1	123
A2A3B7B18DR2DR6	1	0	20
A2A3B7B44DRB111XXDRB10701	1	4	125
A2A3B7B49	1	18	X
A2A3B7B60DR4DR15	1	3	344
A2A7B40B40DR4DR4	1	0	X
A2A9B44B44DR4DR7	1	8	0
A3A11B7B14DR1	1	0	X
A3A11B7B18DR2DR9	1	0	0
A3A11B7B7DR2DR2	1	11	0
A3A23B18B44DR7	1	0	X
A3A23B7B44DR7DR7	1	0	66
A3A24B18B18DR9DR9	1	0	0
A3A25B7B18DR7	1	3	X
A3A28B14B40DR1DR2	1	0	1
A3A28B14B60DR2DR6	1	0	0
A3A28B18B44DR9	1	0	X
A3A28B7B35	1	3	X
A3A28B7B35DR15	1	3	X
A3A28B7B53DR5	1	0	X
A3A30B13B18	1	0	X
A3A30B13B27DR1	1	0	X
A3A31B14B60DR2DR7	1	0	0
A3A31B60B60DR2DR4	1	0	0
A3A31B60B60DR4DR4	1	0	19
A3A31B7B60DR4DR7	1	3	43
A3A3B13B13	1	0	X
A3A3B7B18DR2DR9	1	0	30
A3A3B7B18DR7	1	1	X
A3A3B7B27DR7	1	0	X
A3A3B7B7DR2	1	53	X
A3A3B7B7DR7DR15	1	7	166
A3A9B18B44DR7DR9	1	0	0
A9A19B5B40DR4DR5	1	1	0
A9A28B44B35	1	2	X
A9A30B13B35	1	1	X

Table 8.6b; HLA found in the Traveller group but without a match in Irish bone marrow registry

HLA TYPE	Frequency in Travellers	Frequency in IrBMR	Frequency BMDW
A1A2B7B14DR1DR7	18	0	0
A1A1B7B7DR4DR7	11	0	3
A28A31B14B14DR6DR7	10	0	0
A1A1B7B7DR1DR7	5	0	5
A28A31B7B53DR1DR5	5	0	0
A2A3B7B18DR2DR7	5	0	0
A11A31B7B27DR1DR1	4	0	1
A1A1B7B22DR1DR1	4	0	0
A1A3B7B7DR4DR7	4	0	21
A1A3B7B7DR7DR7	3	0	10
A3B24B7B35DR2DR4	3	0	153
A3A28B7B14DR6DR7	3	0	0
A3A3B18B35	3	0	X
A3A9B7B44DR7DR7	3	0	0
A1A1B7B62DR4DR4	2	0	4
A1A28B7B14DR1DR6	2	0	0
A1A2B14B21DR7DR11	2	0	0
A1A2B35B49	2	0	X
A1A2B62B62DR4DR4	2	0	68
A1A2B7B14DR4DR7	2	0	0
A1A31B14B35DR7DR11	2	0	0
A1A3B7B7DR1DR7	2	0	17
A23A25B5B44DR4DR7	2	0	0
A28A29B44B53DR5DR7	2	0	0
A28A30B18B35DR15	2	0	X
A2A28B7B14DR1DR6	2	0	4
A2A2B14B18DR2DR7	2	0	0
A3A11B7B27DR1	2	0	X
A3A25B5B7DR4DR7	2	0	0
A3A28B7B14DR7DR13	2	0	0
A3A30B18B27DR9DR15	2	0	0
A11A28B7B53DR5	1	0	X
A11A32B8B61DR3DR15	1	0	0
A2A12B8B44	1	0	X
A1A11B8B14DR6DR6	1	0	2
A1A1B7B15DR4DR4	1	0	0
A1A1B7B62DR1DR4	1	0	5
A1A1B7B7DR1DR4	1	0	8
A1A1B7B7DR7	1	0	X
A1A1B7B8DR1DR7	1	0	10
A1A1B8B62DR3DR4	1	0	0
A1A28B14B21DR5DR6	1	0	0
A1A2B14B62DR4DR7	1	0	0
A1A2B7B14DR1DR1	1	0	16
A1A2B7B21DR1DR5	1	0	0
A1A2B7B40DR1DR4	1	0	5
A1A2B8B14DR3DR7	1	0	533
A1A30B8B18DR3DR15	1	0	0
A1A31B7B60DR1DR4	1	0	22
A1A3B21B40DR2DR5	1	0	0
A1A3B7B7DR1DR6	1	0	11
A1A3B7B7DR4DR6	1	0	12
A24A28B44B51DR13	1	0	X

Table 8.6b *continued*

HLA TYPE	Frequency in Travellers	Frequency in IrBMR	Frequency BMDW
A28A28B44B53DR5DR7	1	0	X
A28A30B13B27	1	0	X
A2A19B40B40DR4DR4	1	0	6
A2A23B27B44DR1DR7	1	0	163
A2A25B5B27DR1DR4	1	0	66
A2A28B14B14DR6DR7	1	0	0
A2A28B14B53DR3DR5	1	0	0
A2A2B14B18DR1DR2	1	0	5
A2A2B44B49DR4DR13	1	0	97
A2A30B13B27DR1	1	0	X
A2A3B18B27DR1	1	0	X
A2A3B35B60DR4DR15	1	0	6
A2A3B7B18DR2DR6	1	0	20
A2A7B40B40DR4DR4	1	0	X
A3A11B7B14DR1	1	0	X
A3A11B7B18DR2DR9	1	0	0
A3A23B18B44DR7	1	0	X
A3A23B7B44DR7DR7	1	0	66
A3A24B18B18DR9DR9	1	0	0
A3A28B14B40DR1DR2	1	0	1
A3A28B14B60DR2DR6	1	0	0
A3A28B18B44DR9	1	0	X
A3A28B7B53DR5	1	0	X
A3A30B13B18	1	0	X
A3A30B13B27DR1	1	0	X
A3A31B14B60DR2DR7	1	0	0
A3A31B60B60DR2DR4	1	0	0
A3A31B60B60DR4DR4	1	0	19
A3A3B13B13	1	0	X
A3A3B7B18DR2DR9	1	0	30
A3A3B7B27DR7	1	0	X
A3A9B18B44DR7DR9	1	0	0

X, not tested.

Table 8.6c HLA types found in Travellers but without a match on "Traveller registry" or IrBMR

HLA TYPE	Frequency in Travellers	Frequency in IrBMR	Frequency BMDW
A11A28B7B53DR5	1	0	X
A11A32B8B61DR3DR15	1	0	0
A2A12B8B44	1	0	X
A1A11B8B14DR6DR6	1	0	2
A1A1B7B15DR4DR4	1	0	0
A1A1B7B62DR1DR4	1	0	5
A1A1B7B7DR1DR4	1	0	8
A1A1B7B7DR7	1	0	X
A1A1B7B8DR1DR7	1	0	10
A1A1B8B62DR3DR4	1	0	0
A1A28B14B21DR5DR6	1	0	0
A1A2B14B62DR4DR7	1	0	0
A1A2B7B14DR1DR1	1	0	16
A1A2B7B21DR1DR5	1	0	0
A1A2B7B40DR1DR4	1	0	5
A1A2B8B14DR3DR7	1	0	533
A1A30B8B18DR3DR15	1	0	0
A1A31B7B60DR1DR4	1	0	22
A1A3B21B40DR2DR5	1	0	0
A1A3B7B7DR1DR6	1	0	11
A1A3B7B7DR4DR6	1	0	12
A24A28B44B51DR13	1	0	X
A28A28B44B53DR5DR7	1	0	X
A28A30B13B27	1	0	X
A2A19B40B40DR4DR4	1	0	6
A2A23B27B44DR1DR7	1	0	163
A2A25B5B27DR1DR4	1	0	66
A2A28B14B14DR6DR7	1	0	0
A2A28B14B53DR3DR5	1	0	0
A2A2B14B18DR1DR2	1	0	5
A2A2B44B49DR4DR13	1	0	97
A2A30B13B27DR1	1	0	X
A2A3B18B27DR1	1	0	X
A2A3B35B60DR4DR15	1	0	6
A2A3B7B18DR2DR6	1	0	20
A2A7B40B40DR4DR4	1	0	X
A3A11B7B14DR1	1	0	X
A3A11B7B18DR2DR9	1	0	0
A3A23B18B44DR7	1	0	X
A3A23B7B44DR7DR7	1	0	66
A3A24B18B18DR9DR9	1	0	0
A3A28B14B40DR1DR2	1	0	1
A3A28B14B60DR2DR6	1	0	0
A3A28B18B44DR9	1	0	X
A3A28B7B53DR5	1	0	X
A3A30B13B18	1	0	X
A3A30B13B27DR1	1	0	X
A3A31B14B60DR2DR7	1	0	0
A3A31B60B60DR2DR4	1	0	0
A3A31B60B60DR4DR4	1	0	19
A3A3B13B13	1	0	X
A3A3B7B18DR2DR9	1	0	30
A3A3B7B27DR7	1	0	X
A3A9B18B44DR7DR9	1	0	0

Table 8.7 likelihood of finding a matched donor using different registries

	Number	No match traveller Registry	No match IrBMR	No Match BMDW	Without match on any Registry
All HLA types (complete & incomplete)	211	133 (63%)	85 (40%)	N/A	54 (25%)
Complete 6 antigen HLA type	129	78 (60.4%)	65 (50%)	62 (48%)	16 (13%)

Key: IrBMR, Irish bone marrow registry; BMDW, International bone marrow registry; N/A, not applicable.

Table 8.8 HLA types found only in the traveller group and number of times they occur within the Traveller group

Ranking of frequency within the Traveller group	HLA TYPE	Frequency in Travellers	Frequency in IrBMR	Frequency BMDW
1	A1A2B7B14DR1DR7	18	0	0
3	A28A31B14B14DR6DR7	10	0	0
7	A28A31B7B53DR1DR5	5	0	0
7	A2A3B7B18DR2DR7	5	0	0
8	A1A1B7B22DR1DR1	4	0	0
9	A3A28B7B14DR6DR7	3	0	0
	A3A9B7B44DR7DR7	3	0	0
10	A1A28B7B14DR1DR6	2	0	0
	A1A2B14B21DR7DR11	2	0	0
	A1A2B7B14DR4DR7	2	0	0
	A1A31B14B35DR7DR11	2	0	0
	A23A25B5B44DR4DR7	2	0	0
	A28A29B44B53DR5DR7	2	0	0
	A2A2B14B18DR2DR7	2	0	0
	A3A25B5B7DR4DR7	2	0	0
	A3A28B7B14DR7DR13	2	0	0
	A3A30B18B27DR9DR15	2	0	0

Table 8.9 HLA type without a match in the Traveller, Irish or International Bone Marrow Registry

HLA TYPE	Frequency in Travellers	Frequency in IrBMR	Frequency BMDW
A11A32B8B61DR3DR15	1	0	0
A1A1B7B15DR4DR4	1	0	0
A1A1B8B62DR3DR4	1	0	0
A1A28B14B21DR5DR6	1	0	0
A1A2B14B62DR4DR7	1	0	0
A1A2B7B21DR1DR5	1	0	0
A1A30B8B18DR3DR15	1	0	0
A1A3B21B40DR2DR5	1	0	0
A2A28B14B14DR6DR7	1	0	0
A2A28B14B53DR3DR5	1	0	0
A3A11B7B18DR2DR9	1	0	0
A3A24B18B18DR9DR9	1	0	0
A3A28B14B60DR2DR6	1	0	0
A3A31B14B60DR2DR7	1	0	0
A3A31B60B60DR2DR4	1	0	0
A3A9B18B44DR7DR9	1	0	0

Table 8.10 Ranking of Traveller HLA occurrence in the Traveller group and comparison with the number of times they occur in other groups

RANK	HLA TYPE	Frequency in Travellers	Frequency in IrBMR	Frequency BMDW
1	A1A2B7B14DR1DR7	18	0	0
2	A1A1B7B7DR4DR7	11	0	3
3	A28A31B14B14DR6DR7	10	0	0
4	A2A3B7B14DR7DR7	9	1	0
5	A3A3B7B60DR2DR7	8	2	0
6	A1A3B7B35DR7DR11	6	1	23
7 (6)	A1A1B7B7DR1DR7	5	0	5
	A1A3B7B8DR3DR15	5	97	0
	A1A3B7B8DR3DR7	5	13	0
	A28A31B7B53DR1DR5	5	0	0
	A2A28B8B49DR3DR4	5	4	0
	A2A3B7B18DR2DR7	5	0	0
8 (6)	A11A31B7B27DR1DR1	4	0	1
	A1A1B7B22DR1DR1	4	0	0
	A1A3B7B7DR4DR7	4	0	21
	A2A2B7B44C5C7DR2DR7	4	11	305
	A32A32B44B61DR15DR15	4	1	0
	A3A32B7B44DR15DR15	4	3	27
9 (8)	A1A2B8B62DR3DR4	3	36	0
	A1A3B7B7DR7DR7	3	0	10
	A1A3B7B8DR2DR6	3	7	23
	A2A3B7B27DR1DR7	3	2	66
	A3A11B7B8DR3DR15	3	2	0
	A3B24B7B35DR2DR4	3	0	153
	A3A28B7B14DR6DR7	3	0	0
	A3A9B7B44DR7DR7	3	0	0
10 (26)	A1A1B7B62DR4DR4	2	0	4
	A1A1B7B8DR3DR15	2	15	0
	A1A28B7B14DR1DR6	2	0	0
	A1A29B7B44DR7DR15	2	2	65
	A1A2B14B21DR7DR11	2	0	0
	A1A2B62B62DR4DR4	2	0	68
	A1A2B7B14DR4DR7	2	0	0
	A1A31B14B35DR7DR11	2	0	0
	A1A3B7B7DR1DR13	2	1	24
	A1A3B7B7DR1DR7	2	0	17
	A23A25B5B44DR4DR7	2	0	0
	A28A29B44B53DR5DR7	2	0	0
	A2A24B14B51DR7DR13	2	2	0
	A2A24B44B51DR11DR13	2	1	97
	A2A28B7B14DR1DR6	2	0	4
	A2A28B8B44DR3DR13	2	4	0
	A2A2B12B18DR2DR4	2	5	6
	A2A2B14B18DR2DR7	2	0	0
	A2A31B7B14DR1DR2	2	1	0
	A2A3B7B40DR1DR2	2	2	42
	A2A3B7B7DR7DR7	2	4	33
	A3A24B7B35DR4DR7	2	39	38
	A3A25B5B7DR4DR7	2	0	0
	A3A28B7B14DR7DR13	2	0	0

Table 8.10 *continued*

RANK	HLA TYPE	Frequency in Travellers	Frequency in IrBMR	Frequency BMDW
10(26)	A3A30B18B27DR9DR15	2	0	0
	A3A32B7B61DR15DR15	2	2	17
11 (78)	A11A28B27B44DR1DR7	1	1	95
	A11A32B8B61DR3DR15	1	0	0
	A1A11B8B14DR3DR6	1	1	13
	A1A11B8B14DR6DR6	1	0	2
	A1A1B7B15DR4DR4	1	0	0
	A1A1B7B62DR1DR4	1	0	5
	A1A1B7B7DR1DR4	1	0	8
	A1A1B7B8DR1DR3	1	4	4
	A1A1B7B8DR1DR7	1	0	10
	A1A1B7B8DR3DR4	1	4	0
)	A1A1B8B57DR3DR7	1	48	0
	A1A1B8B62DR3DR4	1	0	0
	A1A24B8B35DR3DR4	1	1	0
	A1A28B14B21DR5DR6	1	0	0
	A1A28B8B14DR1DR13	1	1	0
	A1A29B8B44DR3DR7	1	79	>400
	A1A29B8B44DR7DR14	1	1	142
	A1A2B14B35DR7DR11	1	2	0
	A1A2B14B62DR4DR7	1	0	0
	A1A2B7B14DR1DR1	1	0	16
	A1A2B7B18DR2DR4	1	1	0
	A1A2B7B21DR1DR5	1	0	0
	A1A2B7B40DR1DR4	1	0	5
	A1A2B7B62DR1DR4	1	3	55
	A1A2B7B62DR4DR4	1	3	71
	A1A2B8B12DR3DR4	1	140	121
	A1A2B8B14DR2DR3	1	1	0
	A1A2B8B14DR3DR7	1	0	533
	A1A30B8B18DR3DR15	1	0	0
	A1A31B7B60DR1DR4	1	0	22
	A1A3B21B40DR2DR5	1	0	0
	A1A3B7B60DR1DR2	1	1	0
	A1A3B7B7DR1DR6	1	0	11
	A1A3B7B7DR4DR6	1	0	12
	A1A3B8B27DR3DR9	1	1	0
	A1A3B8B60DR3DR15	1	2	0
	A1A9B5B7DR1DR5	1	1	0
	A29A31B7B44DR1DR7	1	3	15
	A2A11B7B44DR1DR2	1	3	6
	A2A19B40B40DR4DR4	1	0	6
	A2A23B27B44DR1DR7	1	0	163
	A2A25B5B27DR1DR4	1	0	66
	A2A28B14B14DR6DR7	1	0	0
	A2A28B14B53DR3DR5	1	0	0
	A2A2B14B14DR7DR7	1	2	0
	A2A2B14B18DR1DR2	1	0	5
	A2A2B14B40DR4DR7	1	1	13
	A2A2B14B44DR7DR11	1	3	0
	A2A2B14B44DRB10701DRB111XX	1	3	49

Table 8.10 *continued*

RANK	HLA TYPE	Frequency in Travellers	Frequency in IrBMR	Frequency BMDW
11(78)	A2A2B14B62DR4DR7	1	1	0
	A2A2B44B49DR4DR13	1	0	97
	A2A2B7B40DR2DR4	1	5	93
	A2A2B7B7DR2DR4	1	2	192
	A2A3B35B60DR4DR15	1	0	6
	A2A3B7B12DR2DR4	1	49	215
	A2A3B7B14DR6DR7	1	1	8
	A2A3B7B14DRB10701DRB10701	1	1	18
	A2A3B7B14DRB10701DRB11501	1	11	90
	A2A3B7B18DR2DR2	1	1	123
	A2A3B7B18DR2DR6	1	0	20
	A2A3B7B44DRB111XXDRB10701	1	4	125
	A2A3B7B60DR4DR15	1	3	344
	A2A7B40B40DR4DR4	1	0	N/A
	A2A9B44B44DR4DR7	1	8	0
	A3A11B7B18DR2DR9	1	0	0
	A3A11B7B7DR2DR2	1	11	0
	A3A23B7B44DR7DR7	1	0	66
	A3A24B18B18DR9DR9	1	0	0
	A3A28B14B40DR1DR2	1	0	1
	A3A28B14B60DR2DR6	1	0	0
	A3A31B14B60DR2DR7	1	0	0
	A3A31B60B60DR2DR4	1	0	0
	A3A31B60B60DR4DR4	1	0	19
	A3A31B7B60DR4DR7	1	3	43
	A3A3B7B18DR2DR9	1	0	30
	A3A3B7B7DR7DR15	1	7	166
	A3A9B18B44DR7DR9	1	0	0
	A9A19B5B40DR4DR5	1	1	0

Key: (n), number of individuals with that HLA type

Table 8.11 Number of times the patient HLA Type occurs and comparison with other groups

Patient No.	HLA type	Frequency in patient group	Fr in TR	Fr in IrBMR	Fr in BMDW
1	A2A7B40B40DR4DR4	1	1	0	x
2	A1A3B7B7DR1DR6	1	1	0	11
3	A2A3B7B14DR6DR7	1	1	1	8
4	A1A2B7B14DR1DR7	2	18	0	0
5	A2A2B14B14DR7DR7	1	1	2	0
6	A3A28B14B60DR2DR6	1	1	0	0
7	A1A2B7B62DR1DR4	1	1	3	55
8	A2A2B14B62DR4DR7	1	1	1	0
9	A1A2B14B35DR7DR11	1	1	2	0
10	A2A28B14B53DR3DR5	1	1	0	0
11	A2A31B7B14DR1DR2	1	2	1	0
12	A1A29B7B44DR7DR15	1	2	2	65
13	A1A30B8B18DR3DR15	1	1	0	0
14	A2A3B7B18DR2DR7}	2	5	0	0
15	A2A3B7B18DR2DR7}	2	5	0	0
16	A2A3B7B14DR7DR7	1	9	1	0
17	A1A28B7B14	1	5	3	x
18	A1A3B7B7DR1DR7	1	2	0	17
19	A2A3B18B40	1	2	1	x
20	A2A2B7B44CW5CW7DR2DR7	1	4	11	305
21	A1A3B7B8DR2DR6	1	3	7	23
22	A1A1B7B7DR4DR7	1	2	0	3
23	A2A3B7B12DR2DR4	1	1	49	215
24	A1A2B7B14DR1DR7	2	18	0	0
25	A1A28B7B14DR1DR6	1	2	0	0
26	A3A24B7B35DR2DR4	1	3	0	153
27	A2A28B7B14DR1DR6	1	2	0	4
28	A9A19B5B40DR4DR5	1	1	1	0
29	A1A3B7B7DR1DR13	1	2	1	24
30	A1A28B27B49	1	1	1	x
31	A3A28B7B44	1	2	11	X
32	A2A3B7B27DR1DR7	1	3	2	66
33	A23A25B5B44DR4DR7	1	2	0	0
34	A2A24B44B51DR11DR13	1	2	1	97
35	A2A28B44B44DR11	1	2	6	x
36	A2A2B7B44CW5CW7	1	4	x	95
37	A2A24B5B44CW1CW5	1	7	x	16
38	A2A29B44B44	1		118	x
39	A2A28B8B44DR3DR13	1	2	4	0
40	A2A28B8B49DR3DR4	1	5	0	0
41	A3A11B7B8DR3DR15	1	2	2	0
42	A3A32B7B61DR15DR15	1	2	2	17
43	A1A24B8B35DR3DR4	1	1	1	0
44	A3A28B7B14DR7DR13	1	2	0	0
45	A3A3B7B7DRB10701DRB11501	1	1	7	166
46	A2A2B14B44DR10701DRB111XX	1	1	3	49

Key: Fr, Frequency

Table 8.12 Ranking of Traveller HLA types according to their occurrence in the group

Rank	HLA TYPE	Frequency in Traveller group	Frequency in IrBMR	Frequency BMDW
1	A1A2B8B12DR3DR4	1	140	121
2	A1A3B7B8DR3DR15	5	97	0
3	A1A29B8B44DR3DR7	1	79	>400
4	A2A3B7B12DR2DR4	1	49	215
5	A1A1B8B57DR3DR7	1	48	0
6	A3A24B7B35DR4DR7	2	39	38
7	A1A2B8B62DR3DR4	3	36	0
8	A1A1B7B8DR3DR15	2	15	0
9	A1A3B7B8DR3DR7	5	13	0
10 (3)	A2A2B7B44C5C7DR2DR7	4	11	305
	A2A3B7B14DRB10701DRB11501	1	11	90
	A3A11B7B7DR2DR2	1	11	0
11	A2A9B44B44DR4DR7	1	8	0
12 (2)	A1A3B7B8DR2DR6	3	7	23
	A3A3B7B7DR7DR15	1	7	166
13 (2)	A2A2B12B18DR2DR4	2	5	6
	A2A2B7B40DR2DR4	1	5	93
14 (6)	A1A1B7B8DR1DR3	1	4	4
	A1A1B7B8DR3DR4	1	4	0
	A2A28B8B44DR3DR13	2	4	0
	A2A28B8B49DR3DR4	5	4	0
	A2A3B7B44DRB111XXDRB10701	1	4	125
	A2A3B7B7DR7DR7	2	4	33
15 (9)	A1A2B7B62DR1DR4	1	3	55
	A1A2B7B62DR4DR4	1	3	71
	A29A31B7B44DR1DR7	1	3	15
	A2A11B7B44DR1DR2	1	3	6
	A2A2B14B44DR7DR11	1	3	0
	A2A2B14B44DRB10701DRB111XX	1	3	49
	A2A3B7B60DR4DR15	1	3	344
	A3A31B7B60DR4DR7	1	3	43
	A3A32B7B44DR15DR15	4	3	27
16 (11)	A1A29B7B44DR7DR15	2	2	65
	A1A2B14B35DR7DR11	1	2	0
	A1A3B8B60DR3DR15	1	2	0
	A2A24B14B51DR7DR13	2	2	0
	A2A2B14B14DR7DR7	1	2	0
	A2A2B7B7DR2DR4	1	2	192
	A2A3B7B27DR1DR7	3	2	66
	A2A3B7B40DR1DR2	2	2	42
	A3A11B7B8DR3DR15	3	2	0
	A3A32B7B61DR15DR15	2	2	17
	A3A3B7B60DR2DR7	8	2	0
17 (22)	A11A29B27B44DR1DR7	1	1	95
	A1A11B8B14DR3DR6	1	1	13
	A1A24B8B35DR3DR4	1	1	0
	A1A28B8B14DR1DR13	1	1	0
	A1A29B8B44DR7DR14	1	1	142
	A1A2B7B18DR2DR4	1	1	0
	A1A2B8B14DR2DR3	1	1	0

Table 8.12 continued

Rank	HLA TYPE	Frequency in Traveller group	Frequency in IrBMR	Frequency BMDW
17(22)	A1A3B7B35DR7DR11	6	1	23
	A1A3B7B60DR1DR2	1	1	0
	A1A3B7B7DR1DR13	2	1	24
	A1A3B8B27DR3DR9	1	1	0
	A1A9B5B7DR1DR5	1	1	0
	A2A2B14B40DR4DR7	1	1	13
	A2A2B14B62DR4DR7	1	1	0
	A2A31B7B14DR1DR2	2	1	0
	A2A3B7B14DR6DR7	1	1	8
	A2A3B7B14DR7DR7	9	1	0
	A2A3B7B14DRB10701DRB10701	1	1	18
	A2A3B7B18DR2DR2	1	1	123
	A32A32B44B61DR15DR15	4	1	0
	A9A19B5B40DR4DR5	1	1	0
18 (64)	A11A31B7B27DR1DR1	4	0	1
	A11A32B8B61DR3DR15	1	0	0
	A1A11B8B14DR6DR6	1	0	2
	A1A1B7B15DR4DR4	1	0	0
	A1A1B7B22DR1DR1	4	0	0
	A1A1B7B62DR1DR4	1	0	5
	A1A1B7B62DR4DR4	2	0	4
	A1A1B7B7DR1DR4	1	0	8
	A1A1B7B7DR1DR7	5	0	5
	A1A1B7B7DR4DR7	11	0	3
	A1A1B7B8DR1DR7	1	0	10
	A1A1B8B62DR3DR4	1	0	0
	A1A28B14B21DR5DR6	1	0	0
	A1A28B7B14DR1DR6	2	0	0
	A1A2B14B21DR7DR11	2	0	0
	A1A2B14B62DR4DR7	1	0	0
	A1A2B62B62DR4DR4	2	0	68
	A1A2B7B14DR1DR1	1	0	16
	A1A2B7B14DR1DR7	18	0	0
	A1A2B7B14DR4DR7	2	0	0
	A1A2B7B21DR1DR5	1	0	0
	A1A2B7B40DR1DR4	1	0	5
	A1A2B8B14DR3DR7	1	0	533
	A1A30B8B18DR3DR15	1	0	0
	A1A31B14B35DR7DR11	2	0	0
	A1A31B7B60DR1DR4	1	0	22
	A1A3B21B40DR2DR5	1	0	0
	A1A3B7B7DR1DR6	1	0	11
	A1A3B7B7DR1DR7	2	0	17
	A1A3B7B7DR4DR6	1	0	12
	A1A3B7B7DR4DR7	4	0	21
	A1A3B7B7DR7DR7	3	0	10
	A23A25B5B44DR4DR7	2	0	0
	A28A29B44B53DR5DR7	2	0	0
	A28A31B14B14DR6DR7	10	0	0
	A28A31B7B53DR1DR5	5	0	0
	A2A19B40B40DR4DR4	1	0	6
	A2A23B27B44DR1DR7	1	0	163

Table 8.12 continued

Rank	HLA TYPE	Frequency in Traveller group	Frequency in IrBMR	Frequency BMDW
18(64)	A2A25B5B27DR1DR4	1	0	66
	A2A28B14B14DR6DR7	1	0	0
	A2A28B14B53DR3DR5	1	0	0
	A2A28B7B14DR1DR6	2	0	4
	A2A2B14B18DR1DR2	1	0	5
	A2A2B14B18DR2DR7	2	0	0
	A2A2B44B49DR4DR13	1	0	97
	A2A3B35B60DR4DR15	1	0	6
	A2A3B7B18DR2DR6	1	0	20
	A3A11B7B18DR2DR9	1	0	0
	A3A23B7B44DR7DR7	1	0	66
	A3A24B18B18DR9DR9	1	0	0
	A3B24B7B35DR2DR4	3	0	153
	A3A25B5B7DR4DR7	2	0	0
	A3A28B14B40DR1DR2	1	0	1
	A3A28B14B60DR2DR6	1	0	0
	A3A28B7B14DR6DR7	3	0	0
	A3A28B7B14DR7DR13	2	0	0
	A3A30B18B27DR9DR15	2	0	0
	A3A31B14B60DR2DR7	1	0	0
	A3A31B60B60DR2DR4	1	0	0
	A3A31B60B60DR4DR4	1	0	19
	A3A3B7B18DR2DR9	1	0	30
	A3A9B18B44DR7DR9	1	0	0
	A3A9B7B44DR7DR7	3	0	0

Table 8.13 Frequency with which Traveller HLA types occur in BMDW

Rank	HLA TYPE	Frequency in Travellers	Frequency in IrBMR	Frequency BMDW
1	A1A29B8B44DR3DR7	1	79	>400
2	A1A2B8B14DR3DR7	1	0	533
3	A2A3B7B60DR4DR15	1	3	344
4	A2A2B7B44C5C7DR2DR7	4	11	305
5	A2A3B7B12DR2DR4	1	49	215
6	A2A2B7B7DR2DR4	1	2	192
7	A3A3B7B7DR7DR15	1	7	166
8	A2A23B27B44DR1DR7	1	0	163
9	A3B24B7B35DR2DR4	3	0	153
10	A1A29B8B44DR7DR14	1	1	142
11	A2A3B7B44DRB111XXDRB10701	1	4	125
12	A2A3B7B18DR2DR2	1	1	123
13	A1A2B8B12DR3DR4	1	140	121
14 (2)	A2A24B44B51DR11DR13	2	1	97
	A2A2B44B49DR4DR13	1	0	97
15	A11A29B27B44DR1DR7	1	1	95
16	A2A2B7B40DR2DR4	1	5	93
17	A2A3B7B14DRB10701DRB11501	1	11	90
18	A1A2B7B62DR4DR4	1	3	71
19	A1A2B62B62DR4DR4	2	0	68
20 (3)	A2A25B5B27DR1DR4	1	0	66
	A2A3B7B27DR1DR7	3	2	66
	A3A23B7B44DR7DR7	1	0	66
21	A1A29B7B44DR7DR15	2	2	65
22	A1A2B7B62DR1DR4	1	3	55
23	A2A2B14B44DRB10701DRB111XX	1	3	49
24	A3A31B7B60DR4DR7	1	3	43
25	A2A3B7B40DR1DR2	2	2	42
26	A3A24B7B35DR4DR7	2	39	38
27	A2A3B7B7DR7DR7	2	4	33
28	A3A3B7B18DR2DR9	1	0	30
29	A3A32B7B44DR15DR15	4	3	27
30	A1A3B7B7DR1DR13	2	1	24
31 (2)	A1A3B7B35DR7DR11	6	1	23
	A1A3B7B8DR2DR6	3	7	23
32	A1A31B7B60DR1DR4	1	0	22
33	A1A3B7B7DR4DR7	4	0	21
34	A2A3B7B18DR2DR6	1	0	20
35	A3A31B60B60DR4DR4	1	0	19
36	A2A3B7B14DRB10701DRB10701	1	1	18
37 (2)	A1A3B7B7DR1DR7	2	0	17
	A3A32B7B61DR15DR15	2	2	17
38	A1A2B7B14DR1DR1	1	0	16
39	A29A31B7B44DR1DR7	1	3	15
40 (2)	A1A11B8B14DR3DR6	1	1	13
	A2A2B14B40DR4DR7	1	1	13
41	A1A3B7B7DR4DR6	1	0	12
42	A1A3B7B7DR1DR6	1	0	11
43 (2)	A1A1B7B8DR1DR7	1	0	10
	A1A3B7B7DR7DR7	3	0	10

Table 8.13 continued

Rank	HLA TYPE	Frequency in Travellers	Frequency in IrBMR	Frequency BMDW
44 (2)	A1A1B7B7DR1DR4	1	0	8
	A2A3B7B14DR6DR7	1	1	8
45 (4)	A2A11B7B44DR1DR2	1	3	6
	A2A2B12B18DR2DR4	2	5	6
	A2A3B35B60DR4DR15	1	0	6
46 (4)	A1A1B7B62DR1DR4	1	0	5
	A1A1B7B7DR1DR7	5	0	5
	A1A2B7B40DR1DR4	1	0	5
	A2A2B14B18DR1DR2	1	0	5
47 (3)	A1A1B7B62DR4DR4	2	0	4
	A1A1B7B8DR1DR3	1	4	4
	A2A28B7B14DR1DR6	2	0	4
48	A1A1B7B7DR4DR7	11	0	3
49	A1A11B8B14DR6DR6	1	0	2
50 (1)	A11A31B7B27DR1DR1	4	0	1
	A3A28B14B40DR1DR2	1	0	1
51 (61)	A11A32B8B61DR3DR15	1	0	0
	A1A1B7B15DR4DR4	1	0	0
	A1A1B7B22DR1DR1	4	0	0
	A1A1B7B8DR3DR15	2	15	0
	A1A1B7B8DR3DR4	1	4	0
	A1A1B8B57DR3DR7	1	48	0
	A1A1B8B62DR3DR4	1	0	0
	A1A24B8B35DR3DR4	1	1	0
	A1A28B14B21DR5DR6	1	0	0
	A1A28B7B14DR1DR6	2	0	0
	A1A28B8B14DR1DR13	1	1	0
	A1A2B14B21DR7DR11	2	0	0
	A1A2B14B35DR7DR11	1	2	0
	A1A2B14B62DR4DR7	1	0	0
	A1A2B7B14DR1DR7	18	0	0
	A1A2B7B14DR4DR7	2	0	0
	A1A2B7B18DR2DR4	1	1	0
	A1A2B7B21DR1DR5	1	0	0
	A1A2B8B14DR2DR3	1	1	0
	A1A2B8B62DR3DR4	3	36	0
	A1A30B8B18DR3DR15	1	0	0
	A1A31B14B35DR7DR11	2	0	0
	A1A3B21B40DR2DR5	1	0	0
	A1A3B7B60DR1DR2	1	1	0
	A1A3B7B8DR3DR15	5	97	0
	A1A3B7B8DR3DR7	5	13	0
	A1A3B8B27DR3DR9	1	1	0
	A1A3B8B60DR3DR15	1	2	0
	A1A9B5B7DR1DR5	1	1	0
	A23A25B5B44DR4DR7	2	0	0
	A28A29B44B53DR5DR7	2	0	0
	A28A31B14B14DR6DR7	10	0	0
	A28A31B7B53DR1DR5	5	0	0
	A2A24B14B51DR7DR13	2	2	0
	A2A28B14B14DR6DR7	1	0	0

Table 8.13 continued

Rank	HLA TYPE	Frequency in Travellers	Frequency in IrBMR	Frequency BMDW
51(61)	A2A28B14B53DR3DR5	1	0	0
	A2A28B8B44DR3DR13	2	4	0
	A2A28B8B49DR3DR4	5	4	0
	A2A2B14B14DR7DR7	1	2	0
	A2A2B14B18DR2DR7	2	0	0
	A2A2B14B44DR7DR11	1	3	0
	A2A31B7B14DR1DR2	2	1	0
	A2A3B7B14DR7DR7	9	1	0
	A2A3B7B18DR2DR7	5	0	0
	A2A9B44B44DR4DR7	1	8	0
	A32A32B44B61DR15DR15	4	1	0
	A3A11B7B18DR2DR9	1	0	0
	A3A11B7B7DR2DR2	1	11	0
	A3A11B7B8DR3DR15	3	2	0
	A3A24B18B18DR9DR9	1	0	0
	A3A25B5B7DR4DR7	2	0	0
	A3A28B14B60DR2DR6	1	0	0
	A3A28B7B14DR6DR7	3	0	0
	A3A28B7B14DR7DR13	2	0	0
	A3A30B18B27DR9DR15	2	0	0
	A3A31B14B60DR2DR7	1	0	0
	A3A31B60B60DR2DR4	1	0	0
	A3A3B7B60DR2DR7	8	2	0
	A3A9B18B44DR7DR9	1	0	0
	A3A9B7B44DR7DR7	3	0	0
	A9A19B5B40DR4DR5	1	1	0

Table 8.14 Traveller HLA types not found on IrBMR or BMDW

HLA TYPE	Frequency in Travellers	Frequency in IrBMR	Frequency BMDW
A11A32B8B61DR3DR15	1	0	0
A1A1B7B15DR4DR4	1	0	0
A1A1B7B22DR1DR1	4	0	0
A1A1B8B62DR3DR4	1	0	0
A1A28B14B21DR5DR6	1	0	0
A1A28B7B14DR1DR6	2	0	0
A1A2B14B21DR7DR11	2	0	0
A1A2B14B62DR4DR7	1	0	0
A1A2B7B14DR1DR7	18	0	0
A1A2B7B14DR4DR7	2	0	0
A1A2B7B21DR1DR5	1	0	0
A1A30B8B18DR3DR15	1	0	0
A1A31B14B35DR7DR11	2	0	0
A1A3B21B40DR2DR5	1	0	0
A23A25B5B44DR4DR7	2	0	0
A28A29B44B53DR5DR7	2	0	0
A28A31B14B14DR6DR7	10	0	0
A28A31B7B53DR1DR5	5	0	0
A2A28B14B14DR6DR7	1	0	0
A2A28B14B53DR3DR5	1	0	0
A2A2B14B18DR2DR7	2	0	0
A2A3B7B18DR2DR7	5	0	0
A3A11B7B18DR2DR9	1	0	0
A3A24B18B18DR9DR9	1	0	0
A3A25B5B7DR4DR7	2	0	0
A3A28B14B60DR2DR6	1	0	0
A3A28B7B14DR6DR7	3	0	0
A3A28B7B14DR7DR13	2	0	0
A3A30B18B27DR9DR15	2	0	0
A3A31B14B60DR2DR7	1	0	0
A3A31B60B60DR2DR4	1	0	0
A3A9B18B44DR7DR9	1	0	0
A3A9B7B44DR7DR7	3	0	0

Table 8.15 Haplotype frequency in Traveller group

Rank	Haplotype	Occurrence in T group	T Hapolotype frequency	Haplotype frequency IrBMR*(%)
1	A3B7DR7	62	49%	<0.5
2	A2B14DR7	44	35%	<0.5
3	A1B7DR1	39	31%	<0.5
4	A1B7	30	24%	1.3
5	A2B14	29	23%	0.6
6	A1B7DR7	28	22%	<0.5
7	A1B8DR3	27	21%	11.4
8	A1B7DR4	25	20%	<0.5
9	A28B14DR6	21	16.6%	<0.5
10	A28B44	17	13.5%	0.7
11	A2B44	17	13.5%	9.2
12	A3B7	17	13.5%	8.5
13	A3B7DR15	17	13.5%	<0.5
14	A3B7DR2	15	12%	5.3
15	A2B18DR2	14	11%	<0.5
16	A31B14DR7	13	10%	<0.5
17	A2B44CW5	12	9%	N/A
18	A31B7DR1	12	9%	<0.5
19	A32B44DR15	12	9%	<0.5
20	A32B61DR15	12	9%	<0.5
	A3B60DR2	11	8.7%	<0.5
	A24B35DR4	10	8%	<0.4
	A28BW53DR5	10	8%	<0.5
	A2B27DR1	10	8%	<0.5
	A30B13	10	8%	<0.5
	A1B8	9	7.1%	14.3
	A11B27DR1	8	6.3%	<0.5
	A1B35DRW11	8	6.3%	<0.5
	A28B14	8	6.3%	<0.5
30	A2B62DR4	8	6.3	<0.5
	A3B18	8	6.3	<0.5
	A1B62DR4	7	5.5	<0.5
	A28B8DR3	7		<0.5
	A2B35	7		0.7
	A25B5DR4	6	4.7	<0.5
	A2B14DR1	6		<0.5
	A2B49DR4	6		<0.5
	A3B35	6		0.56
	A3B40	6		<0.5
40	A11B8DR3	5	4%	<0.5
	A23B44DR7	5		<0.5
	A24B51DR13	5		<0.5
	A24B5CW1	5		N/A
	A25B51DR4	5		<0.5
	A2B40DR4	5		0.76
	A2B44DR11	5		<0.5
	A2B49	5		<0.5
	A2B7CW7	5		N/A
	A2B7DR1	5		<0.5
50	A30B18DR15	5		<0.5

Table 8.15 continued

Rank	Haplotype	Traveller frequency	T Haplotype frequency	Haplotype frequency IrBMR*(%)
51	A3B18DR9	5		<0.5
52	A9B44DR7	5	4	<0.5
53	A1B7DRB110501	4	3.1	<0.5
	A1B8DR6	4		<0.5
	A1BW22DR1	4		<0.5
	A28B35	4		<0.5
	A29B44DR7	4		<0.5
	A2B12DR4	4		<0.5
	A2B13	4		0.82
60	A2B14DR2	4		<0.5
	A2B44CW7DR7	4		1.1
	A2B7	4		4.9
	A2B7CW5DR2	4		2.1
	A2B8	4		1.3
	A31B60DR4	4		<0.5
	A3B40DR2	4		<0.5
	A3B7DR6	4		<0.5
	A9B35	4		0.85
	A11B7DR2	3	2.3%	0.99
70	A1B21DR5	3		<0.5
	A1B49	3		<0.5
	A28B14DR13	3		<0.5
	A29B44DRB10701	3		<0.5
	A2B14DRB10701	3		<0.5
	A2B44DR13	3		<0.5
	A3B27DR9	3		<0.5
	A3B7DRB10701	3		<0.5
	A10B5	2	1.6%	<0.5
	A11B14DR6	2		<0.5
80	A11B62	2		<0.5
	A11B7	2		1.3
	A19B40DR4	2		<0.5
	A1B14	2		0.79
	A1B15DR4	2		<0.5
	A1B221DRW11	2		<0.5
	A1B8DR7	2		0.65
	A28B27	2		<0.5
	A2B18	2		0.8
90	A2B44DRB111XX	2		<0.5
	A2B7DR2	2		2.1
	A2B7DR7	2		1.0
	A3B13	2		<0.5
	A3B60DR15	2		<0.5
	A3B7DR13	2		0.54
	A3B7DRB11501	2		<0.5
	A3B8DR3	2		<0.5
	A9B5DR5	2		<0.5
	AW24B5CW1	2		N/A
100	A1B57DR7	1	0.7%	<0.5
	A1B60DR1	1		<0.5
	A1B8DR1	1		<0.5

Table 8.15 continued

Rank	Haplotype	Traveller frequency	T Haplotype frequency	Haplotype frequency IrBMR*(%)
103	A1B8DRB11401/7/8	1		<0.5
	A24B18DR9	1		<0.5
105	A24B35	1	0.7%	<0.5
	A24B5CW1	1		N/A
	A25B18	1		<0.5
	A25B44	1		<0.5
	A28B14DR1	1		<0.5
110	A28B40	1		<0.5
	A29B44DR3	1		<0.5
	A29B7	1		<0.5
	A2B12	1		<0.5
	A2B15	1		2.1
	A2B27	1		1.4
	A2B44DR1	1		<0.5
	A2B44DR4	1		3.9
	A2B60DR4	1		<0.5
	A2B7DR4	1		<0.5
120	A2BW44CW5	1		N/A
	A3A35DR11	1		<0.5
	A3B14	1		1.5
	A3B27	1		<0.5
	A3B60	1		<0.5
	A3B60DR4	1		<0.5
126	A7B40DR4	1		<0.5

* From Finch et al 1997

Table 8.16 Patient haplotype frequencies, comparison with various groups

Rank	haplotype	Frequency P group	Frequency Traveller group
1	A1B7DR1	7	39
2	A2B14DR7	5	44
3	A3B7DR7	4	62
4	A2B44CW5	2	12
5	A1B8DR3	2	25
6	A3B7DR15	2	17
7	A3B7DR2	2	15
8	A2B62DR4	2	8
9	A2B44DR11	2	5
10	A28B8DR3	2	7
	A2B18DR2	2	14
	A2B14DR2	2	4
	A28B14DR6	2	21
	A24B35DR4	2	10
	A1B7	1	30
	A1B49	1	3
	A28B44	1	17
	A28B14	1	8
	A28B27	1	2
20	A2B7CW7	1	5
	A24B5CW1	1	6
	A1B8	1	9
	A2B44	1	17
	A3B7	1	17
	A2B12DR4	1	4
	A3B7DRB11501	1	2
	A2B7CW5DR2	1	4
	A3B40	1	6
	A2B44CW7DR7	1	4
30	A2B44DR13	1	3
	A1B8DR6	1	4
	A3B7DRB10701	1	3
	A28B44	1	17
	A2B40DR4	1	5
	A2B18	1	2
	A11B8DR3	1	5
	A2B44DRB111XX	1	2
	A2B7DR1	1	5
	A19B40DR4	1	2
40	A1B7DR4	1	25
	A2B14DRB10701	1	3
	A3B7DR13	1	2
	A23B44DR7	1	5
	A3B7DR6	1	4
	A3B7DR6	1	4
	A1B7DRB1 1501	1	4
	A1B7DR7	1	28
	A29B44DRB10701	1	3

Table 8.16 continued

Rank	haplotype	Frequency P group	Frequency Traveller group
49	A2B27DR1	1	10
50	A9B5DR5	1	2
	A31B7DR1	1	12
	A32B61DR15	1	12
	A3B60DR2	1	11
	A30B18DR15	1	5
	A28B14DR6	1	21
	A28B14DR13	1	3
	A1B35DR11	1	8
	A25B5DR4	1	6
	A24B51DR13	1	5
60	A28B53DR5	1	10
	A7B40DR4	1	1
62	A2B49DR4	1	6

Key; P, patient

Table 8.17 comparison of the commonest occurring Irish Registry HLA phenotypes (1997) with their frequency in this Traveller group.

HLA type	Ranking in IrBMR	Number of times it occurs in Traveller group	Rank in Traveller group
A1A3B7B8DR15DR3	1	5	7
A1A2B8B44(12)DR3DR4	2	5	7
A1-B8-DR3-	3	0	0
A1A11B7B8DR15DR3	4	0	0
A1A2B7B8DR15DR3	5	0	0
A2-B44-DR4-	6	0	0
A1-B8B57DR3DR7	7	1	11
A1A2B8-DR3-	8	0	0
A1A2B8B44DR3DR7	9	0	0
A1A29B8B44DR3DR7	10	1	11

(Finch et al 1997)

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Chapter 9

Closing comments and conclusions.

The Irish Traveller population is in many ways a unique group within the Irish community. It adheres to a community, working class and extended family based collectivism in contrast to the settled community's more middle class, nuclear family based individualism. These socio-cultural differences can lead to inevitable misunderstandings and difficulties in the relationship between the settled and Traveller community. (MacGreil M 1996). Surveys examining attitudes of the settled community towards the traveller population have found that social distance between both communities has increased over a number of years. A survey carried out in 1972-73 and repeated in 1988-89 found that in 1973 29% of a Dublin sample would welcome a traveller as part of the family but this decreased to 16.4% in 1988. Fifty-nine percent of respondents, in 1988, would not have welcomed travellers as next door neighbours and 10% would deny citizenship to Irish Travellers. (Mc Greil, 1996). Reasons cited for this antipathy were "way of life" in 77% of cases and "not socially acceptable" in one fifth of cases. An ESRI 1984 survey found that 76.4% of respondents would be "reluctant to seek out the company of a traveller", 62.9% would exclude a traveller from their circle of friends and 55.9% would avoid a traveller in social situations. (Davis et al, 1984). These attitudes are at the heart of opposition to the provision of serviced halting sites and housing for Travellers. They may also in part explain the 1995 Task force finding that 17% of Travellers found it difficult get a GP and 36% had difficulty getting their GP to do house calls. The Task force found that "GPs tend to be reluctant to accept Travellers as registered patients because of fear that to treat them will result in loss of other patients and because of their high consultation rate"(Task force on the Travelling Community, 1995). All of this has a knock –on effect and may contribute to the Tallaght hospital experience that the majority of Traveller children attending specialist services were referred there by the Accident and Emergency department and not by their GP. The Tallaght study also found that 31% of Traveller children did not receive a discharge letter whereas 100% of settled children did and that 21% of traveller children were referred to clinical support services but 62% of settled children were referred. (Use of Hospital Facilities, 2000). The reasons for this are unclear but the effect is that

potentially important clinical information is not circulated to the relevant health care professionals.

The Task Force on the Travelling community recommended in its 1995 report that " a Traveller Health Advisory Committee should be appointed and should co-ordinate aspects of health status in the Traveller community". It suggested the establishment of a Traveller Health unit to do the same thing at local level. It further recommended an improved system of transferring records within and between health board regions and suggested that a data base of Traveller records might be useful. (Task Force on the Travelling Community, 1995). A progress report was published in 2000. It noted that the Advisory board and Health units had been established. It found that was very little quantitative data on Traveller utilisation of the health services and yet again recommended an improved system of record transfer. It noted that "a nationally centralised compatible record system, which would also facilitate the transfer of records between and within health boards, would be the ideal solution favoured by Traveller organisations". The Department of Health and Children disagreed with these findings and believed that if a "hand-held" patient record system proved feasible then a system of transferring records might not be necessary. (First progress report on the Travelling Community, 2000) Currently neither has been implemented. My data from Chapter 2 demonstrates that Travellers are not identified in the health care system and no specific efforts are being made, even in those areas with the highest concentration of traveller population, to meet their health needs in a cohesive way. My findings in 2001 mirror the Task force on the Travelling communities conclusions in the early 1990s. Some of the problems they highlighted were that:

Health boards were unable to extract data on Traveller utilisation of hospital services due to lack of identification of Travellers on records and due to lack of record keeping.

- All health boards had difficulty in the utilisation and delivery of accident and emergency services which included; "inappropriate use of these facilities, failure to keep recall appointments; threatening behaviour and difficulties arising because Traveller patients are sometimes accompanied by large groups.

- All health boards reported a high uptake of paediatric services but particular difficulties experienced in this area included; overcrowding of the waiting room due to large accompanying groups and discharge against medical advice.

- It was suggested that the health boards would introduce a system of identifying Travellers on Birth, deaths and hospital morbidity collections and that provision would be made to record traveller status during hospital admissions. Such records would be voluntary and should guarantee confidentiality and sensitivity.

The 2000 report could find no evidence of progress in addressing any of these issues. The Department of Health 2002-2005 strategy was to establish a pilot project to collect information on ethnicity which would include travellers and other ethnic groups. There is little evidence that this has been established.

The recommendations on Traveller Health initially made were based on data published in 1987/1988, i.e. already 7-8 years out of date. The Department of Health's published strategy for 2002-2005 is still relying on this data and another study has never been undertaken although there are suggestions that a study is about to be repeated. It is questionable how the current strategy can be audited with any degree of accuracy based on 20 year old data but one hopes that it shows an improvement in Traveller Health.

Consanguinity Issues

Consanguinity is defined as marriage between second cousins or closer. The strategy on Traveller Health 2002-2005 has stated that "cousin marriage is not of itself harmful and does not cause genetic disease. However if both partners carry an altered gene they will be at increased risk of developing genetic disease with on average 1 in 4 children being affected. The Irish Traveller population have chosen to marry within their own communities for successive generations and hence the increase in the prevalence and incidence of hereditary conditions" (Dept of Health, 2002).

The Traveller Consanguinity working group was established in 2002 to undertake an in-depth analysis of issues related to consanguinity. This report has not been published but

a resumé of their recommendations was given to me by Professor Andrew Green, Director of the National Genetics unit and can be found in Fig 9.1. The Department of Health made no commitment to implement any of the findings or recommendations of the consanguinity committee. It undertook to examine the possibility of making genetic screening and counselling services available in all health board regions where there was a significant traveller population and promised to make "vigorous efforts" to ensure that Traveller babies would receive the full range of neonatal metabolic screening.

Outcome of the various Health Care strategies.

Hospital care for Travellers seems to have been largely unaltered by these strategies. There may be information and recommendations from the Department of Health but if so, the Medical stake-holders in Traveller Health have not been informed. There is no national data base or even a hand held system of medical note transfer and this seriously disadvantages the traveller community. A system of shared care between district hospitals and tertiary specialist centres would benefit all concerned.

On a more positive note the Traveller-designated Public Health Nurses have been appointed and are providing and education, liaison and advocacy role for their clients. Unfortunately their role has never been explained to many hospital teams and as a result a very useful mechanism to ensure that Travellers attend appointments and understand the working of hospital outpatient and inpatient facilities has been neglected. It seems likely that the public health strategies including basic immunisation and women's health will pay dividends. Recently a series of Traveller health videos were made to promote health and to explain some of the more common diseases such as asthma. These mechanisms could be extended to hereditary diseases such as Hurler syndrome and Fanconi Anaemia.

Study Outcomes

This is the first project to address the prevalence of haematologically-related disease in the Traveller community. There was a need to define these conditions in order to

facilitate their diagnosis and optimal management. It was also essential to examine current practice in order to establish protocols for treatment and follow-up to obtain maximum benefit from treatment strategies.

It was obvious from my initial attempts to define the types of disease in the Traveller population that despite the establishment of various task-forces and working parties there is little knowledge about Traveller conditions outside of the tertiary referral hospitals. This stems from lack of national databases and communication between the tertiary centres and local hospitals. The ambivalent attitudes of one community for the other also has a role to play. There has been a fundamental misunderstanding of the role of hospitals, outpatients, accident and emergency departments and primary health care amongst some members of the Travelling community. The Traveller organisations and Traveller designated Public health nurses have done much to alter this, but for many Travellers hospital is still an alien and intimidating place.

We will need to look at our outpatient structures to better facilitate Traveller attendance. In particular we need to organise an appointment system which will inform the Traveller in question that an appointment has been made, not necessarily an easy task if they are living in non-permanent/non-serviced sites or if their level of literacy means that they cannot read their post, assuming that the postman calls.

Formalised shared care links need to be established between the tertiary centre and the local hospitals. There should be an annual disease up-date meeting comprising a combination of lectures and workshops for all of the health professionals dealing with Traveller child health care.

I have identified and added to the list of congenital disease known to the National Genetics centre. I have also identified the genetic defect in 3 of these conditions. Recently direct sequencing of amplified genomic DNA has facilitated prenatal DNA diagnosis in Carbonic Anhydrase II deficiency whereas the technology had previously been quite cumbersome (Chapter 7) [Shah GN et al, 2004]. The identification of other hereditary diseases and the gene mutations involved will facilitate the National Genetics centre's work.

Evaluation of specific Diseases (Table 9.1)

Seven diseases were identified with haematological links (Chapter 2). Haematopoietic stem cell transplant has a well defined role in 3 conditions; Hurler syndrome, Fanconi Anaemia and Haemophagocytic lymphohistiocytosis. The role of HSCT in Carbonic Anhydrase II deficiency is currently unclear. This study has analysed the data relating to HSCT from 1984 - 2002.

Hurler syndrome (Chapter 6)

Stem cell Transplantation for Hurler syndrome has modified the disease but the majority of children remain developmentally delayed and have progressive dysostosis multiplex. In the past there was a tendency to search for an extended family donor for these children however my data suggests that speed of transplant may improve outcome. Other studies have suggested that transplant should be undertaken in children less than 24 months of age (Staba SL et al, 2004; Peters et al, 1998), whereas my data would suggest that transplantation in children less than 1 year of age may be of maximum benefit (Chapter 6). This means that families at risk for Hurler syndrome should be identified and early neonatal screening, possibly using a technique similar to that described by Chamoles, should be undertaken (Chamoles et al, 2001). This will facilitate rapid referral for transplant assessment. Enzyme replacement therapy may be of benefit to prevent Glycosaminoglycan build-up in the pre-transplant period, which should be as short as possible (Grewal et al, 2005). Community-directed cord banking could expedite transplantation as even a 3 HLA locus mismatch has been associated with successful engraftment (Staba et al, 2004).

Fanconi Anaemia (Chapter 4)

Fanconi Anaemia (FA) is the commonest haematological disease in the Traveller population but until this study the natural history of FA in the Traveller community was unknown. I have demonstrated that "classical" FA is due to a large deletion of exons 11-14 in FANCA gene. Bone marrow failure is inevitable and, unlike most FANCA genotypes, occurs between 40-65 months. It can be associated with early development

of MDS/AML and this seems associated with early onset bone marrow failure (<40 months). Some children with FA were referred for the first time to the Paediatric Haematology Service when they developed bone marrow failure. This is unacceptable and all children with FA should be referred within the first year of life and potential donors identified. Some of the children with FA were treated for a period of time as "failure to thrive" before they were diagnosed. I would recommend that all Traveller children from "at risk" groups should have clastogenic stress testing preformed as part of their "failure to thrive" work-up. It appears that HSCT is effective in the management of bone marrow failure (Alter et al, 1998), but there is a continued increased risk of head and neck cancers suggesting that long-term regular ENT monitoring is required (Kutler et al, 2003).

A second group of FA has also been identified. These have a complete absence of Natural Killer cells and 2 of the group have developed haemophagocytic lymphohistiocytosis. It is as yet unclear if this group will develop bone marrow failure but the data available suggests that they do not. It is unlikely that this group, who are all from the same FA kindred as those with "classical" FA, will have another breakage syndrome. Nevertheless this must be considered. (New et al, 2005)

Haemophagocytic Lymphohistiocytosis (Chapter 5)

This is a rare condition which has presented on 3 occasions in Traveller Children. It has been associated on all 3 occasions with NK deficiency. Perforin abnormalities have been documented in one of the children, the other 2 were not tested. All 3 children have had classical presentations but whilst 2 of the children had a relatively protracted course the third child had very aggressive symptoms which failed to respond to immune modulation. Traveller children presenting with a constellation of symptoms including prolonged fever and hepatosplenomegaly should be referred for evaluation of possible HLH using the schema recommended by Arico. (Arico et al, 2002). Those with fulminant disease should be treated aggressively, possibly with Etoposide-containing regimens and transplanted as a matter of urgency. (Hirst et al, 1994; Chen et al, 1998; Durken et al, 1999). An unrelated/community cord bank would facilitate this management strategy.

Carbonic Anhydrase II deficiency (Chapter 7)

The role of HSCT in this condition is, as yet, unclear. In many parts for the world Carbonic Anhydrase II deficiency (CAII) is a relatively mild condition not requiring HSCT. Our group has an aggressive form leading to severe developmental delay and visual disturbance. Stem cell transplantation initially appeared beneficial. (Mc Mahon et al, 2001). Recent evidence of continued optic nerve damage following transplant had brought about a re-evaluation and suspension of HSCT in this group. Nevertheless longer follow-up may lead to reinstitution of HSCT as a definitive treatment. If this proves to be the case, it is imperative that the donor has a normal CA2 genotype and that the transplant is undertaken as soon as possible following birth, preferably before 3 months, to prevent optic nerve damage.

Factor X Deficiency (Chapter 3)

Severe factor X deficiency in the Traveller group is caused by a novel mutation, Pro382Leu, which disrupts folding and prevents protein formation. (Deam et al, 2004). It produces a severe bleeding phenotype, commonly presenting with life-threatening bleeds within the first 72 hours of life. Regular prophylactic factor X replacement therapy has been shown to reduce bleeding episodes but individual variability in the half-life of factor X means that fall-off studies are vital to avoid catastrophic bleeding. (Machin et al, 1980; McMahon et al, 2002). Early central venous catheter placement will facilitate these prophylactic regimens and surgery can be performed safely using Prothrombin Complex. (McMahon et al, 2000; McMahon et al, 2002).

Conclusions

This study has helped define the types of inherited disease existing in the Traveller population and has evaluated current management strategies for those diseases presenting to the Haematologist. It is clear that the Traveller population have specific social needs which must also be evaluated and addressed as they may have a significant bearing on outcomes e.g. lack of permanent housing, problems availing of primary

health care. Certain Kindreds have specific genetic conditions and efforts should be made to inform them about these conditions, possibly using video or audio tape as literacy should not be assumed. Neonatal screening for specific diseases such as Hurler syndrome should be undertaken and children referred to tertiary Haematology units as soon as possible. Those children with metabolic disease amenable to HSCT should have this performed without delay.

The issue of community directed cord banking is quite difficult to untangle. My data exploring the benefits of a dedicated Traveller stem cell bank has shown that it could potentially provide 15% of children with a donor. The published data suggests that it also facilitates earlier transplantation. (Chapter 8) It is difficult to see how it could be established for one community and not for other minority groups and as such needs to be considered in the context of a universal cord banking policy. There may be a role for directed family banking but such cords, would have to be genetically tested to ensure that they were not heterozygous for the condition.

Fig 9.1 Recommendations of the Traveller Consanguinity Working Group

1. Traveller couples who are first cousins should be informed of their general risk and offered voluntary genetic counselling
2. The primary tool to assess risk is a detailed family history
3. With a documented family history of a genetic disorder, a couple may be offered, in a non-directive manner, gene carrier tests
4. There is no impediment to first cousin marriages
5. A more detailed study of consanguinity in Travellers is needed.
6. Resources should be made available to Traveller communities, Traveller organisations and Traveller Health workers to raise awareness of consanguinity and to pilot educational programmes fro health workers and for Travellers
7. More resources are needed for the National Centre for Medical Genetics and for the National Centre for Inherited Metabolic Diseases
8. Awareness should be raised for Health Care Workers in general.

(With thanks to Prof. Andrew Green)

Table 9.1 Resumé of Haematologically-associated Disease in Traveller Community

Type of Disease (*Gene*)	Number of affected Individuals	Treatment	Outcome	Recommendations
Hurler syndrome *(W402X)*	31	Supportive	Death <10years	Early diagnosis ?Enzyme replacement Rapid identification of BM donor
		BMT	Survival Dysostosis Multiplex unaffected Developmentally delayed Cataracts	Definitive treatment Transplant <1year; preferably under 6 months Genotypically normal donor ? Role of Enzyme replacement pre-transplant
Fanconi Anaemia *(FANCA Del 11-14)*	19	Supportive	Death once BM Failure/Acute Leukaemia develops	Early diagnosis Early identification of donor Limit blood products
		Androgens	Improvement in Blood counts Virilisation	Avoid if possible
		BMT	Definitive treatment Continued risk of cancer	Definitive treatment/successful outcome Long-term care plan should include regular head & neck evaluation
HLH	3	Immune-modulation	Relapse	Early diagnosis Aggressive management
		BMT	Cure	Definitive treatment
CAII deficiency *(Del_{12}/ins_4 Exon 6)*	6	Supportive	Fractures Developmental delay Loss of vision	Intensive supportive care Psychological support Educational support
		BMT	Cure of osteopetrosis Reduction in Cerebral calcification Curtailment of visual disturbance ? improvement on developmental delay	? Transplantation within the first 6 months ? Genotypically normal donor
FX Deficiency *(Pro382Leu)*	4	FX replacement	Prevention of Intracranial bleeding	Primary prophylaxis administered after birth to prevent ICH
		Prophylaxis	Reduction in bleeding episodes and joint damage	Insertion of CVAD Commence Prophylaxis once/twice/week "Fall-off" studies to ensure correct dosing regimen

Key: BMT, bone marrow Transplantation; HLH, haemophagocytic lymphohistiocytosis; CAII, Carbonic anhydrase II deficiency; FX, Factor Ten; ICH, Intracranial Haemorrhage; CVAD, Central Venous Access Device.

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Appendices

Appendix 1

Department of Paediatric Haematology

21/6/01

Dr M. Barry
The Chairperson AMNCH Ethics Committee,
National Centre for Pharmocoeconomics,
St James Hospital,
Dublin 8

RE: Study of inherited Haematological Disease in the Travelling Community

Dear Dr Barry,

I, under the supervision of Dr Owen Smith and Dr Emer Lawlor, propose to study inherited haematological disease within the Travelling Community and am unsure if this study should be submitted for formal ethical approval. I would therefore appreciate your guidance.

The Irish Travelling Community is a distinct social grouping within the Irish population who has long campaigned to have their cultural differences recognised. They are also a socially deprived group who has health issues which have not been seen in the settled community for 40 years. There is an increased incidence of autosomal inherited disease within the Travelling Community due to the high degree of consanguineous marriage. These types of disease are almost completely unknown in the settled community but have been seen in other culturally separate communities.

The major part of the study involves the collation of data from patients who attend Dr Smith and who have inherited haematological disease. We plan to define the diseases involved, the outcome of treatments given and the genetic mutations involved. All of this evaluation is part of our standard practice but it is the first time that the data will be formally collected for the Travelling population. Gene analysis if not required to make the diagnosis is undertaken only after obtaining informed consent (form enclosed).

Many of the autosomal recessive diseases found within the Travelling Community may be fatal within the first years of life or lead to irreparable damage if not rapidly treated by gene therapy in the form of stem cell transplant. Stem cells may be obtained from either bone marrow or cord blood (taken at the time of delivery). These stem cells must be HLA compatible i.e. they must be of the same type as the recipient. The second part of the study looks at HLA types in the Travelling community, the Irish bone marrow donor bank and the world wide donor bank. This data is recorded on the databases of the Irish Blood Transfusion service. No extra samples are required and the individuals concerned are identified by unique patient numbers. The aim of this part of the study is to identify if unique or very rare HLA types occur in the Travelling Community because of their social diversity which will make it less likely for them to find a donor within the short time frame available for transplant. If this proves to be the case then newer strategies need to be implemented to facilitate this community.

No published data exist on the incidence and types of inherited disease within the Travelling Community. I propose to attempt to define this by questionnaire of all the Paediatricians within the Republic of Ireland. I do not wish to identify these patients and do not wish to be able to contact them but simply to determine the types of disease. I enclose the questionnaire and accompanying letter for your examination.

I would again emphasise that this study does not seek to identify individuals, undertake clinical trials or subject any individuals to blood sampling or procedures which would not be part of the normal standards of care for that disease. All of the parents and also the older children are aware of our interest and have been most supportive of it.

I would appreciate your advice on this project.

Many thanks,

Yours sincerely,

Corrina Mc Mahon. MB, DCH, MRCP(UK), MRCPath
Paediatric Haematology Co-ordinator.

Appendix 2

THIS NOTEPAPER MUST NOT BE USED FOR PRESCRIPTIONS OR INVOICING PURPOSES

Dan Lynch. Joint Research Ethics Committee Secretariat.
Telephone : 4142860. Fax : 4142371. Email: dan.lynch@amnch.ie

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Dr. O. P. Smith,
Consultant Paediatric Haematologist,
Adelaide and Meath Hospital, Dublin
incorporating The National Children's Hospital,
Tallaght,
Dublin 24.

3rd September, 2001.

RE : **Study of Haematological Disease in the Travelling Community**

Please quote this reference in all communications regarding this study : 010811/13801

Dear Dr. Smith,

The Joint Research Ethics Committee at its meeting on 28th August 2001 agreed to give ethical approval to the above study subject to the following conditions :

1. The letter to paediatricians should be more specific in relation to the initial disorders;

2. The Investigator is asked to indicate how this study links in with the recent Eastern Regional Health Authority study in this area;

3. The Investigator is asked to provide a definition of "inherited disorders" in the context of this study.

4. The Committee perceived the Consent Form to be confusing and the Investigator is asked to redraft it in a clearer style.

5. The Committee queries whether the stated objectives can be achieved in the current setting.

Yours sincerely,

Daniel R. Lynch,
Senior Executive Officer.

cc Dr. Corrina McMahon.

Appendix 3

Department of Paediatric Haematology
30/1/02

Mr D. Lynch,
Senior Executive Officer,
Joint Research Ethics Committee Secretariat,
AMNCH,
Tallaght,
Dublin 24

RE: **Study of Haematological Disease in The Traveller Community.**
Ref: 010811/13801

Dear Mr Lynch,

Thank you for your letter re the above study. I have considered the points the committee have raised and address them below. I hope this will prove satisfactory.

1. Redrafted letter to Paediatricians and new questionnaire are enclosed

2. How does this study link with ERHA study in this area?

The work of the Traveller Health unit is a work in progress. Their first report highlights areas of specific difficulty:

- Consanguinity – leading to the emergence of Metabolic disorders, however there is no information on the incidence, prevalence or any comprehensive study of the types of disorder occurring in the Traveller population and this lack of information inhibits the development of a specific genetic councilling service.
- The Travellers lack information which will facilitate the appropriate utilization of the services available to them in hospitals
- The children's health strategy focuses on Sudden Infant Death Syndrome, Asthma, immunisations and child development

My study aims to define the types of inherited disorders existing in the Traveller community and it should also define of the prevalence of these disorders, what types of disorders exist and the outcome for these diseases. This will be the first information of this kind to be assembled on a 26 county basis and will facilitate the work of the National centre for Genetic Studies with whom I will liaise. It should form the basis for other studies in the area. Many of the disorders I will study have developmental implications, I will assess the therapeutic options for these children and this should be of interest to the child health strategy group. This information will also beneficial to paediatricians and indeed I have received great encouragement from the Paediatrician group about this work.

3. Definition of Inherited disorders:

The commonest inherited diseases in the Traveller group are inherited in an autosomal recessive fashion. These disorders/diseases arise because one or both parents have one abnormal or defective gene which they pass on to the child. This means that the child has 2 copies of a particular gene which are defective or faulty and hence has the disease. The parents are said to be carriers of the disease and are usually not affected. Two of the 4 grandparents will also be carriers and many of the relatives may also be carriers. This situation most commonly occurs if there is a consanguineous marriage

4. Redrafted consent form is enclosed

5. Can the stated objectives be achieved in the current setting?

The stated objectives can certainly be achieved and will add to the body of information about this group. In some instances it will provide information which has not previously been known. I cannot be certain that this information will prompt a change in Health Board strategy but if the information is never obtained there will be no impetus to change.

I hope this answers the questions raised. If you require any further information I can be contacted by e mail: CORRINA@iol.ie, or through the department.

Many Thanks,

Yours sincerely,

Corrina McMahon, MB, DCH, MRCP(UK), MRCPath

Appendix 4

THIS NOTEPAPER MUST NOT BE USED FOR PRESCRIPTIONS OR INVOICING PURPOSES

Dan Lynch, Joint Research Ethics Committee Secretariat.
Telephone : 4142860. Fax : 4142371. Email: dan.lynch@amnch.ie

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Dr. Corrina McMahon,
Dept of Paediatric Haematology,
Adelaide and Meath Hospital, Dublin
incorporating The National Children's Hospital,
Tallaght, Dublin 24.

18th February, 2002.

RE : Study of Haematological Disease in the Travelling Community

Please quote this reference in any follow-up to this letter : 010811 / 13801 / 2002 / 1 / 8

Dear Dr. McMahon,

Thank you for your letter dated 30/1/02 in which you respond to the conditions attached to the initial ethical approval of the above study. This response has been approved by the Chairman on behalf of the Joint Research Ethics Committee. Ethical approval for this study is now complete.

Yours sincerely,

Daniel R. Lynch,
Senior Executive Officer.

Appendix 5

Survey of Inherited Disease in the Traveller Community

I have □ / have not □ patients who meet the description.

If yes, please continue, if not please go to Q9 and return the form in the envelope provided.

Q1. Patient's Initials…… Q2. Age/DOB……...

Q3. Type of Disease:

Haematological □ Metabolic □ Immunodeficiency □ Other □

Q4. Name of Disease:

……

Q5. Other Disease(s) occurring in this patient:

……..
……..
……..

Q6. Age at diagnosis:

Birth □, within 1st yr □, 1-2yrs □, 2-3yrs □, 3-4yrs □, >4yrs □

Q7. Treatment Received (Diet, symptomatic, BMT, gene therapy):

……
……
……

Q8. Consultant(s) Attended:

……
……
……
……

Q9. Name/Address of person completing this form:

……..

Appendix 6

Survey of Inherited Haematological Disease in the Traveller Population

I do ☐ / do not ☐ have patients with inherited haematological disease.

If you have patients please continue, if not please go to Q11 and return the form in the envelope provided.

Q1. Patient's initials…….. Q2. Age/DOB…….

Q3. Name of disease:

……..

Q4. Age at diagnosis: ……..

Q5. Reason for diagnosis:

……
……
……
……

Q6. Age at first symptoms:……

Q7. Type of symptom:

……
……
……
……

Q8. Treatment(s) received: *{please tick all appropriate boxes}*

Type of treatment	Date of/age at 1st treatment
☐ Blood transfusion	……..
☐ Platelet transfusion	……..
☐ Factor Concentrate	……..
☐ Bone Marrow Transplant	……..
☐ Umbilical cord/stem cell Transplant	……..
☐ Gene Therapy	……..
☐ Androgens	……..
☐ Other: *(please list)*	
……..	…… *(PTO)*

Survey of Inherited Haematological Disease in the Traveller Population (II)

Q9. Reason for BMT/Stem Cell Transplant *(if undertaken)*:

.....
.....
.....
.....
.....
.....

Q10. Outcome of Disease *(please tick relevant box)*:

☐ Dead:

Cause.....
.....

☐ Alive/asymptomatic/Not recieving treatment:

.....
.....

☐ Alive/asymptomatic/Receiving treatment:

Please explain.....
.....
.....

☐ Alive/symptomatic/Not receiving treatment:

Please explain:.....
.....
.....

☐ Alive/symptomatic/Receiving treatment:

Please explain.....
.....
.....

Q11. Name/address of person completing this form:

.....
.....
.....
E-Mail: .....

Appendix 7

Consent Form for Factor X Gene Analysis

Factor X is a very important clotting factor at the heart of clot formation. People with factor X deficiency have a lower than normal level of factor X. This can lead to some bleeding problems such as nose bleeds, bruising or bleeding more than usual following surgery. Some people do not have any factor X at all and may experience very severe bleeding problems.

Factor X deficiency is a rare disorder and because of this very little is known about why it occurs and why some people may have more bleeding problems than others. We are undertaking a study of Factor X with the help of colleagues in London. We plan to look at the gene for factor X to try to discover what is wrong with it. We also plan to look at how factor X works and see if we can say why a particular gene abnormality causes a particular type of bleeding problem. This will enable us to learn a lot more about Factor X deficiency.

Your child/children and perhaps you are known to have Factor X deficiency and we would be grateful if you would allow us to take a blood sample from your child/children and yourself to help us carry out this research. We will need to take about 10-20 mls of blood. Some of this blood will be sent to London and the remainder will be used in our research Laboratory. Unfortunately we cannot guarantee that our research will be successful and we also cannot guarantee that even if it is successful we will have results within any particular timeframe. However if any meaningful results do emerge we will give these to you so that you can know more about your Factor X deficiency.

If you agree to having this blood test done for research purposes, we would ask you to sign the consent form below. You do not have to agree to this and if you do not wish to have blood taken this refusal will not in any way alter either your or your child's management.

Consent Form

I ..... have read the information provided and have had an opportunity to ask Dr..... questions about this study. I understand that I do not have to consent to this and that my/my child's treatment will not be compromised if I do not agree.

I consent to my/my child's .....
blood being taken for this study . I understand that no useful information may emerge from this study.

Signed..... Date.....
Doctor's signature..... Date.....
Witness Signature..... Date.....