Dedication

I dedicate my work to my parents and family, who have always believed in me and gave me their support and unconditional love. Thank you for believing in me and enlighten my life when I am down. Thank you for your support, inspiration and your always being there for me when I am in need.

Special thanks to my country, UAE and our government who without their support I would not reached this far.

Thanks to my colleges back home who build up my confidence, ambition and advancement in my medical carrier.

I cannot finish without remembering my father and wished he was here with us to feel the joy of his daughter. Dad I dedicate my work to you and mom. May you rest in peace.

Abstract

Background:

Sepsis is a systemic illness caused by microbial invasion of normally sterile parts of the body. In haematological malignancies, patients are more prone to developing infections due to defects in the neutrophil count and function which occur as a part of the neoplastic process or due to chemotherapy. The presence of neutropenia calls for other means of defence including the innate immune system.

Genetic studies have attempted to examine the relationship between particular genes involved in innate immunity and susceptibility to infections. Genes involved in the host defence mechanism such as pathogen presentation, recognition and phagocytosis result in the initiation of a cascade of events ending in the innate immune system activation. Chitotriosidase and nucleotide oligmerization domain (NOD2) are two genes suggested to have a possible role in the innate immune response against bacterial and fungal infections. Studies of acute lymphoblastic leukaemia (ALL) and NOD2 mutations have been conducted in allogenic transplant patients in order to examine any association with the incidence of relapse, survival and graft versus host disease. The occurrence of NOD2 variants are also implicated in the onset or progression of different malignancies via its effect on immune system.

Purpose gene:

The aim of our study was to explore the effect of mutations in genes involved in the innate immune system in relation to incidence and outcome of sepsis, prevalence of particular microorganism, and depth and duration of neutropenia in patients with haematological malignancies. The study aimed to identify mutations in chitotriosidase and NOD2, individually, and then looked at the synergetic effect of

both mutations, given previous evidence of molecular interaction between the gene products. We also looked at NOD2 mutation in non-transplanted ALL patients so as to evaluate the effect of this mutation on outcome and prognosis.

Methods:

The study was carried out in the RFH in patients diagnosed with haematological malignancies. Blood was collected and DNA extracted. Genotyping identified chitotriosidase mutations while NOD2 missense mutations (SNP8 and SNP12) were determined by pyrosequencing.

In the study of NOD2 mutations and ALL outcome, samples were collected as a part of UKALL-12 trial-MRD study in patients with the diagnosis of ALL. The NOD2 mutations (SNP8, SNP12 and SNP13) were identified by genescanning.

Results:

The incidence of febrile events with positive (p=0.031) or negative growth for any organism cultures (p=0.029) was increased in patients with chitotriosidase mutations. These results were most significant during periods of neutropenia; febrile events with bacterial isolates (p=0.015) and without (p=0.007).

During periods of normal neutrophil count the incidence of fevers with positive bacterial cultures was also increased in patients with NOD2 mutation,(p=0.017). The incidence of fevers without positive cultures was decreased (p=0.029). There were no significant differences in CR, incidence of relapse or OS in ALL patients with NOD2 mutations.

Conclusions:

This study suggests an association between chitotriosidase mutations and the incidence of febrile events (with or without positive bacterial cultures) in neutropenic patients. The NOD2 mutation was found in association with an increased incidence of fevers with bacterial organisms identified and a decreased incidence of fever of unknown origin in non-neutropenic patients.

Acknowledgements

In the process of my project a lot of people gave me a hand in stepping out from difficult moments and helped me accomplish my ambition.

I thank Dr. Derralynn Hughes who gave me a lot of her time, helped in moulding my scientific thinking, and always answering my questions logically

.Professor Letizia Foroni, I am greatly in debt to you for all the help and encouragement. Thanks for opening new avenues for me when I thought there were none. Trust me when I say my laboratory skills would not have improved without your advice, patience and logical explanations. As well this project would not have finished without your care.

Thanks for Pav Rai who worked side by side with me and try to solve any problem rising.Thanks for my colleges Dr. Lena Rai, Mrs. Aditi Dey who their presence eased me a lot. With them I could just ask and they will give a hand whatever the situation was, thanks for your help, advice and laughs. It is great to have friends like you.

Thanks for Mr. Rob Baker, Mrs. Birunthini Naidu, Veronique Duke and John Yaxley for their help and guidance.

Special thanks for Professor James Owen for his help and guidance. Thanks for listening, helping and trying always to uncover the obstacles I came across.

Thanks for the Haematolgy Laboratory for supplying me with the patients' samples help me locate them even when they were busy.

I would like to thank my family members (mom, husband, sisters, brother, brothers in law, nieces and nephews) for their love and support through my stay in the UK.

Lastly I cannot finish without thanking Dr Satish Keshav for his support and advice.

Table of Contents

Chapter   1
GENERAL INTRODUCTION

Chapter   2
GENERAL METHODs

Chapter   3
CHITOTRIOSIDASE as genetic determinant of sepsis in haematological malignancy

Chapter 4
NOD2 as genetic determinant of sepsis in haematological malignancy

Chapter 5
NOD2 mutation and ALL outcome

Chapter   6
Genetic determinant of sepsis in haematological malignancy
Comparative study: NOD2 & Chitotriosidase mutations

Chapter   7
General discussion

List of Figures

List of Tables

Glossary

ACCP/SCCM	American College of Physicians & Society of Critical Care Medicine
AD	Alzheimer's disease
ALL	Acute lymphoblastic leukaemia
Allo	Allogeneic
AMCase	Acidic mammalian chitinase
AML	Acute myeloid leukaemia
ANC	Absolute neutrophil count
BAL	Bronchoalveolar lavage
B-ALL	B-lineage ALL
BCR	BrEak-point cluster region
B/C	Blood culture
bp	Base pair
BS	Blau syndrome
BSI	Blood stream infection
BMTx	Bone marrow transplant
C.	Candida
CARD	Caspase-recruitment domain
CD	Crohn's disease
cDNA	Complementary DNA

CI	Confidence Interval
Cl.	*Clostridium*
CLL	Chronic lymphocytic leukaemia
CML	Chronic myeloid leukaemia
CNS	Central nervous system
CR	Complete remission
CRBSI	Catheter related blood stream infection
CRI	Catheter related infection
CRP	C-Reactive Protein
CSFs	Colony stimulating factors
CT	Computed tomography
CVC	Central venous catheter
dd H_2O	Double distilled water
DFS	Disease free survival
DNA	Deoxyribonucleic acid
E coli	*Escherichia coli*
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme linked immune Sorbent Assay
EoN	Episode of neutropenia
FNE	Febrile neutropenic event
FNnE	Febrile non-neutropenic event

FUO	Fever of unknown origin
GD	Gaucher's disease
GM-CSF	Granulocyte macrophage colony stimulating factor
GvHD	Graft versus host disease
HLA	Human leukocyte antigen
H. pylori	*Helicobacter pylori*
HT CT	High resolution CT
IBD	Inflammatory bowel disease
ICU	Intensive care unit
IFD	Invasive fungal disease
IFN	Interferon
Ig	Immunoglobulin
IKK	IκB kinase
IL	Interleukin
kb	kilobase
kDa	kilo dalton
LDH	Lactase dehydrogenase
LPS	Lipopolysaccharide
LRR	leucine-rich repeat
LS	Lamya Saeed
	The clinical research fellow

MALT	Mucosa associated lymphoid tissue
MDP	Muramyldipeptide
µL	Micro litre
MLL	Mixed-lineage leukaemia
µM	Micro meter
MM	Multiple myeloma
MRD	Minimal residual disease
mRNA	messenger RNA
MS	Multiple sclerosis
NASH	Non alcoholic steatohepatitis
NF-κB	Nuclear factor kappa B
NHL	Non-Hodgkin's lymphoma
NOD2	Nucleotide oligmerization domain
OS	Overall survival
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate-Buffered Saline
PCR	Polymerase chain reaction
PGN	Peptidoglycan
Ph	Philadelphia chromosome (or BCR-ABL fusion gene)
PMN	Polymorphnuclear neutrophil
R	Resistance protein

RFH	Royal free hospital
RICK	Receptor interacting serine/threonine kinase
rpm	Rotations or revolutions per minute
SCT	Stem cell transplant
SLE	Systemic lupus erythematous
SNP	Single nucleotide polymorphism
Spp	Species
T-ALL	T-lineage ALL
TLR	Toll like receptor
TMP-SMZ	Trimethoprim-sulfamethoxazole
TNF	Tumour necrosis factor
TRM	Transplant related mortality
TtCR	Time to achieve complete remission
UK	United Kingdom
USG	Ultrasonography
WBC	White blood cell count
WT	Wild type

Chapter   1

General Introduction

1.1.   Introduction

Neutropenia is known to occur in haematological malignancies either as a complication of the disease process or of chemotherapy.[2] Consequently, neutropenic patients are at increased risk of severe infections.

The body's defences depend on the innate immune system which consists of physical barriers and cells such as monocytes.[3] The effectiveness of these cells is determined by a variety of genes which influence processes such as immune recognition, phagocytosis and cell signalling and include genes such as chitotriosidase and nucleotide oligmerization domain (NOD2). It has been found that mutations in the NOD2 gene influence the ability of the cells to respond to bacterial infections.[4, 5] Likewise recent studies revealed increased susceptibility in chitotriosidase deficient patients to fungal and gram negative organisms.[6, 7] In the present study we explored the relationship between the developments of infection in patients with haematological malignancies who carry mutations in chitotriosidase or NOD2 genes. We then analysed the effect of gene mutations on the process of infection, occurrence of sepsis, prevalence of microorganisms, and the duration and outcome of these events. We also examined the effect of the mutation on the duration of neutropenia.

Since recent studies have shown conflicting results on the effect of NOD2 mutation on allogenic transplant outcome in translocation negative acute lymphoblastic leukaemia (ALL) patients[8-10] we also conducted a pilot study examining whether NOD2 mutations affect disease outcome and prognosis.

1.2. Haemopoietic malignancies

The haemopoietic malignancies are clonal diseases that derive from a single cell in the marrow or peripheral lymphoid tissue which has undergone genetic alteration through a multistage process.[11] The combination of genetic and environmental influences may determine the individual risk of developing malignancy, however in the majority of cases the cause is unknown, and neither a genetic susceptibility nor an environmental agent is apparent.[11, 12] Haemopoietic malignancies are broadly divided into B and T lymphoid and myeloid; and depending upon onset and course, into acute and chronic (see appendix-3).[13]

In this study we examined the impact of NOD2 mutation in the outcome of Philadelphia negative ALL. Additionally, we studied the impact of chitotriosidase and NOD2 mutation first as a risk factor for developing any of the haemopoietic malignancy and then we looked into the risk of contracting infections in the same group of patients.

1.2.1. Aetiology of haemopoietic malignances

1.2.1.1. Inherited factors

Leukaemia is greatly increased in incidence in some genetic syndromes and diseases, either due to chromosomal abnormalities,[14] immunodeficiency[15] or both. Examples include Klinefelter's syndrome,[16] Bloom's syndrome,[14] and Ataxia telangiectasia.[14, 15] Neurofibromatosis type-1 is believed to increase risk of juvenile myelomonocytic leukaemia by 200-500 fold.[17]

Down's syndrome is associated with 10-30 fold increase in the frequency of acute leukaemia.[18] The associations are also present between Wiskott-Aldrich syndrome[15] and lymphoid granulomatosis and Fanconi's anaemia and myelodysplastic syndromes.[14] There is a clear association of enteropathy-type T-cell lymphoma with Coeliac disease.[19]

Familial tendency in haematopoietic malignancies such as acute myeloid leukaemia (AML), chronic lymphocytic leukaemia (CLL), Hodgkin's lymphoma (HL), non-Hodgkin's lymphoma (NHL), and multiple myeloma (MM) have been investigated in several studies where increased risk in some families with estimated range of 1.5-4.0 fold was observed.[20, 21] These observations suggests that a recessive mode of inheritance as well as shared environment during childhood and adolescence may play a role in familial non-Hodgkin lymphoma.[21] The risk of NHL was also increased in subjects with family history of cancers of the liver, breast and kidney.[20]

1.2.1.2.   Environmental influences

Two studies[22, 23] have examined whether exposure to environmental factors vary between individuals with and without family history of hematopoietic cancer. In these studies, homosexual behaviour, drug use, exposure to pesticides and other occupational contaminants, history of liver diseases, and alcohol consumption[22] were more strongly associated with NHL among men with family history of hematopoietic cancer than among those without such history, indicating a possible interaction between genetic susceptibility and some non-genetic risk factors.

Diet had been described in connection with haematological malignancies. Increased risk of NHL was associated with dietary intake of dairy products, fried or red meat, and coffee, and decreased risk with dietary intake of fruits and vegetables.[24] Eating disorders have been connected to leukaemia due to malnutrition and subsequent impaired immunity.[25]

Radiation exposure is associated with all types of leukaemia (chronic myeloproliferative diseases, chronic myelomonocytic leukaemia, acute myeloid leukaemia), and survivors of the atom bomb explosions in Japan are evidence of such association.[26] Chronic exposure to ultraviolet light,[27] trichloroethylene,[28] uranium,[29] pesticides,[30] benzene[31] as well as cigarette smoking,[32] have been linked to increased incidence to NHL, leukaemia and other cancers. Cigarette

smokers have an increased risk of 2-folds in developing AML and myelodysplastic syndromes.[32, 33] An increase was also found for follicular lymphoma among female smokers.[34]

Increased risk of NHL was also seen in conditions leading to immunosuppressant either due to diseases, treatment or both, examples of such are Sjögren's syndrome,[35] organ transplant,[36] inflammatory bowel disease[37, 38] and rheumatoid arthritis.[39] The risk was stronger when the treatment included cyclosporine-A and azathioprine.[36-38] Rheumatoid arthritis has also been linked to the development of IgA myeloma.[40]

Agents involved in the treatment of malignancies can themselves predispose the patient to secondary lymphohematopoietic malignancies. Cytogenic agents may play a part in the causation of chronic myelomonocytic leukaemia, myelodysplastic syndromes and acute myeloid leukaemia. Alkylating agents, melphalan, procarbazine and nitrosoureas predispose individuals to acute myeloid leukaemia, especially if combined with radiation, or if used in the treatment of lymphocytic or plasmacytic disorders. Epipodophyllotoxins such as etoposide, is associated with secondary leukaemias with balanced translocation such as MLL (mixed-lineage leukaemia) 11q23.[14, 41]

1.2.1.3.   Infections

The mechanism by which infection lead to the development of tumours is unclear, but an abnormal immune response to infection has been suggested by epidemiological studies.[42] Viral infection is known to cause several types of haemopoietic malignancies.[43, 44] Epstein-Barr virus (EBV) has been implicated in lymphoma including; endemic Burkitt's lymphoma,[45] post transplant lymphoproliferative disease (PTLD) which develops during immunosuppressive therapy after solid organ transplantation,[36] mature (peripheral) B-cell neoplasms and several others.[42] Hepatitis B virus (HBV),[46] hepatitis C virus (HCV),[47] Helicobacter pylori (*H. pylori*), human immunodeficiency virus (HIV),[48] retrovirus

human type 1[49] and human herpes virus 8[50] are all associated with different types of lymphoma.

1.3. Acute lymphoblastic leukaemia (ALL)

1.3.1. Incidence and Etiology

ALL has a bimodal age distribution[51] being the most common malignancy in childhood and accounting for 30% of all cancers and 80% of all leukemia.[51, 52] Peak incidence is highest in children aged 2-6 years. In adulthood the incidence is fairly low, but rises gradually with half the cases being under 50. ALL is rare over the age of 70.[52] ALL is seen more commonly in males than females. It is 30% higher in boys compared to girls.[52]

Delayed exposure to infectious agents in children of higher socioeconomic status may also predispose them to ALL.[52] Increased risk is also seen after exposure to radiation, e.g. Chernobyl tragedy and in genetic conditions such as Down's syndrome which carries 20 fold increased risk.[52]

Little is known about the aetiology of childhood leukaemia, but is likely to involve an interaction between inherited susceptibility, environment, haemopoietic development and chance. The concept that some cases of ALL originate *in utero* comes from genetic studies of identical twins with concordant leukaemia, with results suggesting transplacental exposure to certain mutagens may be important in initiating events in some cases. But for leukaemia to emerge additional genetic alterations, such as genetic polymorphisms of certain enzymes capable of detoxifying carcinogens, or leukaemia-specific fusion-gene sequences are required.[53, 54]

1.3.2. Pathogenesis and Prognostic factors

1.3.2.1. Prognosis:

Prognosis usually depends on clinical factors including clinical presentation and biological characteristics such as age, performance status, white blood cell (WBC) count, organ involvement, leukaemia cell immunophenotype, cytogenetics and time to achieve complete remission (CR). Tolerance for intensive chemotherapy may contribute to the poor results in adults compared to childhood leukaemia.[55]

ALL is fatal in days or weeks if untreated depending on the extent of bone marrow failure, related blood cytopenias, the effect of circulating blast cells and their metabolites on vital organs and the degree of central nervous system (CNS) involvement.[52]

Complete remission can be achieved in 70-80% of patients. Around 10-20% (depending upon age) of patients die early during induction treatment and a further 10% could be refractory to remission-induction programs. In addition, more than half of the patients who achieve a complete remission are expected to relapse, and only a minority can achieve a second long lasting remission. The overall chance of cure is approximately 20-40% in adults newly diagnosed with ALL.[55]

Presentation age and leukocyte count in B-lineage ALL are the clinical features most consistently associated with prognosis.[54] In general, as patients age increases, the prognosis worsens. Age over 50-60 years is usually considered as unfavourable prognostic indicator with survival not exceeding 0.20 at 3 years. Adolescents and teenagers under 20 years of age do nearly as well as the paediatric ALL population and are best treated on childhood rather than adult programs.[52]

Elevated cell count reflect a higher tumour mass and is a negative prognostic sign depending on the type of study and whether absolute blast count or total white blood cell count was employed.[52]

The time to response and disease sensitivity to treatment (chemo-radiotherapy) is the primary determinants for survival. Patients achieving CR in 4-5 weeks, evidence of slow peripheral blood (day 7) or bone marrow (day 14) blast cells clearing, or a poor prednisone response carry a significant better outcome.[52, 56] Time to platelet recovery (TPR) within 60 days may be another favourable clinical prognostic marker.[52, 56] Among patients with MLL rearranged ALL, infants younger than one year of age fare considerably worse than older children.[53]

1.3.2.2. Immunophenotyping ALL Precursors

ALL accounts for 80% of paediatric leukaemias,[53, 57] comprises of two cell lineages. B-cell lineage comprises approximately 85-90% of ALL, while T-cell lineage account for 10% of all ALL cases.[13] In childhood ALL B-cell lineage account for approximately 75% of cases less than 18 years of age.[58] While T-cell ALL account for 15% with predominance in males beyond 10 years of age.[59] Roughly 75% of adult ALL are of B-cell lineage, while T-cell lineage constitutes approximately 25% of adult ALL cases.[13, 52]

1.3.2.3. Age

Age is a well known prognostic factor in ALL,[60] with initial remission in most patients, but ultimately over 25% of patients will relapse and only 10% of bone marrow relapses will have the chance to survive.[57]

Prognosis in childhood ALL has continuously improved over the last 25 years with CR rate exceeding 90% and the current cure rate being 75-80%.[60] In adults, although CR rates of 65-85% may be achieved,[55] survival rate at 5 years is still between 20 and 40%.[60] ALL response duration is frequently short, with only 20-30% of patients becoming long-term survivors.[55]

1.3.2.4. Gender

Overall males with ALL fare more poorly than females.[59] The male gender influence over mortality has been observed in several studies.[60] including Baccarani M *et al.* in which it is concluded that the association of male gender and advanced age (≥ 30years), were major determinants of higher death rate.[61]

Although the incidence of ALL is 2 folds higher in white children than black children, but the latter are less fortunate in prognosis.[59]

1.3.2.5. WBC count & other blood parameters

The WBC counts reflect early disease progression, this seems particularly important in B-lineage ALL, as long-term remission remains rare in patients with high WBC counts given standard chemotherapy.[60] In patients with B cell precursor ALL, an age of 1-9 years with low WBC count ($<50 \times 10^9$/l) confers a favourable prognosis, while for T cell ALL, age and WBC count have little clinical significance.[59]

Castagnola *et al.* found that the only factor affecting disease free survival (DFS) was a high WBC count at presentation. While shorter overall survival (OS) statistically correlated with higher WBC counts, long time to achieve CR, and older age.[55] Persisting lack of haematopoietic recovery in the bone marrow (BM) even without the presence of leukemic blasts after 7 or 14 days of induction therapy , is highly an indicator of disease recurrence.[59]

Diagnostic lactase dehydrogenase (LDH) level also represents the initial tumour mass and has been shown to have an influence on CR,[60] although a clear impact on overall survival has not yet been demonstrated.[60]

1.3.2.6.   Cytogenetics

Combined immunophenotypic-cytogenic evaluation allows the identification of chromosomal aberrations including the most common chromosomal translocations like t(9;22)/Ph (~25% of patients) and t(4;11)/MLL-AF4 positive (~8% of patients) B-cell ALL.[52] These techniques can also distinguish between minimally differentiated AML, ALL (including identification of B-cell and T-cell ALL) and acute megakaryoblastic leukaemia.[13] Genetic abnormalities are important prognostic factors within precursor B lymphoblastic neoplasms.[62] Cytogenetic aberrations are less frequent in T-lineage ALL, the most frequent involve 14q11 breakpoints.[52]

The Philadelphia chromosome (Ph) is a shortened chromosome 22 generated by a balanced chromosomal translocation t(9;22), which involves the ABL-1 and BCR (Break-point cluster region) fusion genes. Prognosis in ALL Philadelphia positive is generally dismal for adolescents, but is relatively favourable in children 1 to 9 years old with low WBC count at diagnosis.[59] Although complete haematological remission occurs in approximately 70% post chemotherapy induction, few sustain this remission, as most patients relapse early. The long-term survival rate in adult patients does not reach 20%.[63] Recently studies showed that some cytogenetic abnormalities of ALL blasts are the most important long-term survival prognostic factors.[60] Patients with (Ph+) and/or BCR-ABL-1 fusion gene ALL have extremely poor prognosis, with long-term survival rates ranging from 35% to 40% in children to less than 20% in adults.[64] Additionally most patients have other abnormalities such as heterogenous karyotypes. Whether secondary aberrations carry additional pathogenetic or prognostic information is not yet clear.[63]

The single most frequent cytogenetic aberration in childhood ALL is hyperdiploidy (>50 chromosomes), comprising one quarter of all cases and only 6% of all cases in adult ALL.[53, 59]

ALL with chromosomal rearrangements; t(4;11) is rare in adults (3-4%). It is associated with hyperleukocytosis and the presence of the immature B-ALL subtype.[52] Childhood Ph+ ALL or the t(4;11)/MLL-AF4 fusion are considered to

have very high risk ALL and do worse in infants younger than 1 year compared to children 1 year of age or older.[59, 65] Other recurrent chromosomal abnormalities have been described in adult ALL, but with low frequency and unclear prognosis relevance.

ALL B lineage and prognosis are summarised in table 1.1.

Table 1.1. Acute lymphoid leukaemias; Prognostic markers in Precursor B cell (adapted)[13]

Cytogenics	Genetics	Prognosis
t(9;22)(q34;q11.2)	BCR/ABL	Unfavourable
t(4;11)(q21;q23)	AF4/MLL	Unfavourable
t(1;19)(q23;p13.3)	PBX/E2A	Unfavourable
t(12;21)(p12;q22)	TEL/AML1	Favourable
t(10;14), t(14q11-q13)		Favourable
ALL-hypodiploid		Unfavourable
ALL-hypodiploid >50		Favourable
Hyperdiploid		Favourable

1.4.   Therapy related complications in haematological malignancies

1.4.1.   Neutropenia & infections in Haematologic Patients

The leading cause of treatment-related mortality among patients with haematological malignancies undergoing chemotherapy is multi-organ failure due to systemic infection during neutropenia.[66] Most neutropenic patients with infection present with fever as the first symptom. However, there is a variety of causes for febrile conditions. Apart from infection, reactions to drugs or blood products as well as tumour-associated fever are all possible underlying mechanisms.[67]

Neutropenic patients often fail to develop symptoms and signs of infection due to their blunted inflammatory response.[66, 67]

Occasionally, an infection may develop in the absence of fever, as with *Clostridium spp*, or if the patient receiving corticosteroids.[67] Alternatively fever may be the initial

and often the only sign of infection. Approximately 50-60% of febrile neutropenic episodes in neutropenic patients are classified as fever of unknown origin (FUO) as they never have clinical or microbiological evidence of infection.[67]

The duration and severity of neutropenia are both important and influence not only the frequency and severity of infection but also the response to therapy and overall outcome.[67]

Susceptibility to infection increases when the absolute neutrophil count (ANC) falls below 1000 cell/mm^3 and with the further fall of ANC $\leq$ 500 cell/mm^3 the risk becomes greater. Many patients with haematological disorders (e.g. acute leukaemia) have defects in neutrophil function despite adequate or increased numbers of neutrophil. Chemotherapy induced neutropenia is often superimposed on other immunological deficits that might be present because of the underlying malignancy.[67]

Bloodstream infections (BSIs) directly affect clinical course and outcome in patients with cancer, prolonging their hospital stay and significantly increasing mortality.[68] Among patients with underlying cancer diseases, those with haematological malignancies are more prone to infectious complications due to interactions of host/environment/antimicrobial prophylaxis/treatment related factors.[68] Haematologic patients with BSIs still fear poor outcome despite advances in antimicrobial therapy and supportive care.[68, 69]

The incidence of BSIs in neutropenic patients with the diagnosis of FUO ranges from 11% to 38%. The clinical manifestation may range from transient bacteraemia to fulminate septic shock in those patients.[70]

The types of infections that afflict neutropenic patient undergoes periodic changes, geographic and instituitional differences,[67] as seen in the change of pathogens in the last two decades, with the shift of organism spectrum, from Gram-negative bacteria as a cause of approximately 70% of bacteraemia in the 1970s to Gram-positive organisms causing approximately 70% of episodes by the late 1980s and 1990s. Recently some hospitals have experienced an increase in multidrug

resistance pathogens.[66] Gram-negative bacillary catheter-related blood stream infections (CRBSIs) caused by organisms such as *Escherichia coli* (*E coli*), *Klebsiella pneumoniae, Pseudomonas aeruginosa, Enterobacter spp., Acinetobacter spp.*, and *Stenotrophomonas maltophilia* have been reported. Although infections due to Gram-negative organisms usually emerges from a non-catheter-related source, such as nosocomial urinary tract infection, nosocomial pneumonia, or intra-abdominal infection.[71] The skin organisms such as Coagulase-negative *staphylococci* (mainly *Staphylococcus epidermidis*) and the *oral viridans* (or alpha-haemolytic) *streptococci* (principally *Streptococcus mitis* and *Streptococcus oralis*) account for most of the isolates from blood cultures and lead the cause of CRBSIs. The prominence of the *staphylococci* is attributed to the widespread use of indwelling intravenous-access devices which often remain in place for weeks and even months and the association of *Staphylococcus aureus* with high rate of deep-seated metastatic infections, including septic thrombosis and endocarditis. The occurrence of oral mucositis induced by more intensive chemotherapy is associated with bacteraemia due to *viridans streptococci* that normally reside on the surfaces of the oral cavity.[71, 72]

Infections are predominantly bacterial during the early phases of neutropenia. The spectrum of infections change to fungal with prolong neutropenia, and the administration of multiple courses of broad-spectrum antibiotics. Yeasts such as *Candida spp.* and *Trichosporon spp.* and molds such as *Aspergillus spp.*, the *Zygomycetes*, and *Fusarium spp.* are the usual causes of fungal infections.[67]

Viral infections are uncommon, but herpesviruses (HSV, VZV, CMV, HHV6) and community respiratory viruses are the most frequent pathogens.[67]

1.4.2.   Intravascular catheter

Vascular catheters are becoming an essential tool for the care and management of chronically and critically ill patients. Vascular catheter-related infections are the leading cause of nosocomial BSIs and are associated with substantial morbidity

and mortality.[71, 73] Catheter related infections (CRI) are often difficult to treat, as they are mostly caused by organisms resistant to antimicrobial agents that are embedded in the catheter layers and sheath.[73]

1.4.2.1.   Source of infections

The most common source of colonization of intravascular catheters is the skin and catheter hub. In the long term catheters the lumen of the hub or the bell of the port is the major source of infection.[73]

The skin insertion site is the major source of colonization and contamination with *spp.* of Coagulase negative *Staphylococcus*, in the case of short term catheter. Whereby organisms such as *Staphalylococcus aureus*[71] migrate along the external surface of the catheter and the intercutaneous and subcutaneous segments, leading to colonization of the intravascular catheter tip which may lead to bloodstream infection. The microbes that colonize the catheter hubs and the skin surrounding the insertion site are most commonly cause of CRBSIs.[74]

1.4.2.2.   Clinical manifestations

The clinical manifestations of CRIs can be systemic or local, occurring at the insertion site or tunnel track.[71] Signs and symptoms of local catheter infections are often characterized by inflammatory manifestations which are frequently absent in neutropenic patients, signs including induration, erythema, warmth, and pain or tenderness at or around the catheter exit site.[71, 75] Systemic catheter infections present with signs and symptoms of bacteraemia or fungaemia, such as fever, chills, and hypotension.[71] Clinical manifestations of CRIs, such as fever, chills and hypotension,[71] are sensitive but not specific for a diagnosis, whereas local manifestations are specific but not sensitive.[76]

1.4.2.3. Diagnosis

Microbiological evidence is essential in pointing catheters as the source of bloodstream infections and the diagnosis of CRBSIs in patients with neutropenia. Diagnostic approaches can be divided into two major groups: those necessitating catheter removal and those that can be done without the removal of the catheter.[71] A definite diagnosis of intravascular CRIs cannot be complete by clinical criteria alone, but usually removing the catheter for quantitative catheter-tip culture is necessary. Only 15-25% of central venous catheters (CVCs) removed with the suspicion of infection proved to be the actual source of infection.[77]

The differential quantitative blood cultures from samples drawn simultaneously from the catheter and a peripheral vein, is another test which minimize the risks associated with the removal of the old catheter and placement of a new catheter at a new site.[77] Currently the gold standard for the diagnosis of catheter related bloodstream infection and in cases were catheter removal is undesirable, is the quantitative blood culture techniques.[75, 76]

1.4.3. Sepsis

Sepsis describes a complex clinical syndrome that results from a harmful or damaging host response to infection, as a result from systemic inflammatory response (SIR).[78, 79] Sepsis develops when the initial, appropriate host response to an infection becomes amplified, and then dysregulated.[78, 79]

Rapid activation of humoral cascade systems (complement, coagulation, kallikrein-kinin system) and of cells that support the inflammatory reaction (granulocytes, monocytes, lymphocytes, macrophages, endothelial cells) is the key event in pathogenesis of sepsis. Production and release of inflammatory mediators and vasoactive or cytotoxic molecules is the result of such activation[79] (Figure 1.1).

The onset is often insidious: clinical features may include fever, mental confusion, transient hypotension, diminished urine output or unexplained thrombocytopenia.[78, 79]

Figure 1.1. Cell surface recognition of lipopolysaccharide (LPS):
LBP: LPS-binding protein signal via Toll-like receptor 4 (TLR4) after sensing the LPS. LPS is also sensed by other cell surface molecules including: Macrophage scavenger receptor (MSR), CD11b/CD18 and ion channels.
NOD proteins (nucleotide oligomerization domain), are intracellular receptor to bacterial LPS, in response it initiates a cascade that activates NFκB signalling. Figure adapted.[78]

Untreated, failure of renal or respiratory systems, coagulation abnormalities, and profound unresponsive hypotension may develop.[78]

Bacterial motifs called pathogen-associated molecular patterns (PAMPs) and are the microbial components that are recognized by the innate immune system. This recognition initiates injury.[78] In Gram-negative bacteria, lipopolysaccharide (LPS; known also as endotoxin) is a major component of the outer membrane and is

constructed of a lipid bilayer, separated from the inner cytoplasmic membrane by peptidoglycan (PGN). The LPS molecule is embedded in the outer membrane and linked to the hydrophobic lipid portion. The lipid A portion of the molecule serves to anchor LPS in the bacterial cell wall (figure 1.1.).[78] Under physiological conditions changes in the lipid A structure occur which correlate with the ability to activate host cell membranes; the active forms take the shape of truncated cone, whereas the inactive molecules assume a lamellar structure and become progressively more cylindrical.[78]

Gram-positive bacteria have no endotoxin, but do contain PGN and lipoteichoic acid on their cell wall, which account for their biological activity. Both peptidoglycan and lipoteichoic acid are pro-inflammatory and have the ability to bind to cell surface receptors. Although they are much less active than LPS, but can induces septic shock due to their production of potent exotoxins.[78]

1.4.3.1.   Definition of sepsis

The international standard definition of sepsis is the same definition suggested in 1994 by the consensus conference of the American College of Chest Physicians and the Society of Critical Care Medicine (ACCP/SCCM). Sepsis is defined as a systemic inflammatory reaction induced by microorganisms invading the blood stream and/or their toxins released from a focus of infection. Severity, response to treatment and outcome are determined by the presence and extent of organ dysfunction and development of septic shock, which is associated with the worse prognosis. Septic shock is defined by volume refractory hypotension; decrease organ perfusion despite adequate fluid substitution and mental alteration.[79]

1.4.3.2.   Incidence of sepsis

Prospective studies assumed more than 90% of febrile neutropenic episodes fulfil the criteria of sepsis according to the (ACCP/SCCM) consensus definition.

Accordingly, the incidence of neutropenic sepsis secondary to intensive myelosuppressive chemotherapy is around 70-100%.[80] The incidence of sepsis in this group is related to the pre-treatment of the patient, intensity of chemotherapy, the degree and duration of neutropenia and overall performance status.[79] In the majority of studies, the incidence of neutropenic infections due to bacteria has been reported to be 10-30%.[79]

1.4.3.3.   Prognostic factors in leucocytopenic patients with sepsis

Disease-associated defects in the immune defence mechanisms account for the high incidence of invasive infections in patients diagnosed with haematological malignancies. Examples are chronic lymphocytic leukaemia and multiple myeloma which are associated with humoral defects, while Hodgkin's lymphoma are associated with cellular defects.[79]

Other disease or treatment associated risk factors are: The severity and duration of neutropenia following cytotoxic chemotherapy. Skin and mucosal barriers and as a result of invasive diagnostic procedures, and catheters insertion can be disrupted, leading to increased risk of CRBSIs (staphylococci, fungi). Mucosal dryness due to decreased production of saliva or retention of secretion, facilitate the growth of pathogenic microorganisms. Malnutrition, parenteral nutrition, hospitalization, immunosuppression and antibiotic pretreatment all predispose to nosocomial infections.[79]

1.4.4.   Diagnosis

1.4.4.1.   Bacteraemia

Bacterial infections are life threatening in neutropenic patients. It is critical in this set of patients to diagnose or rule out infection. The most common parameter is the

acute phase, C-reactive protein (CRP). This protein concentration is elevated in almost all cases of microbial infections and within 24hrs.[81]

Microbiology laboratory plays a definite and important role in the diagnosis of infections in neutropenic patients. Such an analysis of various biological examinations: blood cultures (B/Cs), throat swabs. Washes from mouth and nose, sputum, saliva and bronchial secretions obtained either by bronchoscopy, bronchalveolar lavage (BAL) or catheters. Methods permitting the diagnosis of an infection by copro-cultures, cytobacteriological examination of urine, samples of respiratory origin, cytobacteriological examination of cerebrospinal fluid...) (see appendix-4; A.4.1.).[82, 83]

Simultaneous quantitative B/Cs; involves obtaining paired blood cultures drawn simultaneously from the CVC and the peripheral vein, this method found to be the most accurate test for diagnosis of CRBSIs (figure 1.2.).[71]

1.4.4.2.   Gastrointestinal infections

Acute abdominal signs and symptoms are seen in up to 5% of all cancer patients undergoing cytostatic treatment. The definite causative organism is only diagnosed in one third. Organisms are Gram-negative aerobes (e.g. *Pseudomonas aeruginosa*, *E. coli*) and anaerobes (e.g. *Clostridium (Cl) spp*), rarely *Candida spp*, *H. simplex* or cytomegalovirus.[3] Enterocolitis accounts for almost 50% of the gastrointestinal infections in neutropenic patients (*neutropenic colitis*) and is the most common. Presentation is with painful abdomen, nausea, subileus or ileus.[3] *Cl. difficile* toxin is identified in 10–45% in patients with diarrhoea after chemotherapy. While Gram-positive cocci and Gram-negative rods (e.g. *E. coli*, *Klebsiella spp*) were grown in the blood of 80% of patients with neutropenic enterocolitis.[3]

Abdominal symptoms and complications such as abscesses, perforation or ascites should have a proper clinical examination, an abdominal X-ray, blood cultures as

well as cultures of faeces including *Cl. difficile* toxin A & B and cultures of *Cl. difficile*. Abdominal computed tomography (CT) scans, abdominal X-rays using a water soluble contrast medium and abdominal sonography (USG). An endoscopy should be considered if no diagnosis was reached. These tests should only be conducted in patients with diarrhoea or suspected *Cl. difficile* associated ileus.[3]

1.4.4.3.   Skin infections

In skin and soft tissue infections, the whole body surface and the visible mucosa need to be examined. If the infection is localized a microbiological diagnosis with an appropriate specimen (swabs, pus, and effusion) can be reached. If systemic infection is suspected, empirical antibiotic regimen should be initiated after obtaining blood cultures. Where fungal infection is suspected a skin biopsy should be tested cytologically, and in treatment refractory disease or uncertain diagnosis Gram staining and culture should be carried out.[3]

Urinary tract infections (UTI) showed no differences in neutropenic or non-neutropenic patients. The diagnosis and treatment follows the same guidelines as for non-neutropenic patients except for empirical antibiotic treatment duration (7-10 days), started immediately after specimens for microbiological analyses have been taken.[3]

1.4.4.4.   Pulmonary fungal infections

Pulmonary infiltrates is a known complication of intensive chemotherapy and occurs in 15-25% of all patients with profound neutropenia. Within five days after the onset of fever they become apparent in almost two third of the patients. Febrile neutropenia associated with pulmonary infiltrates are at particular high risk of mortality. This risk increases if neutropenia is prolonged (>10days) or diagnosis and so treatment are delayed.[83, 84]

The past decade had raised hope for an early non-invasive detection of pulmonary fungal infections with development of new techniques. Techniques based on detection of fragments of fungal cells by molecular methods and *Aspergillus galactomannan* Sandwich ELISA test.[83, 85]

Radiological detection of lung infiltrates by conventional chest radiographs occurs in less than 10% of persistent fevers despite antimicrobial therapy whereas simultaneous CT scans, in particular high resolution techniques, suggest 50% of these patients may have pathological lung findings.[83]

It is recommended in cases of positive chest radiography to perform bronchoscopy with BAL in the affected area of the lung. In order to specify the cause of the infiltrates further, an additional thoracic CT may provide useful information. If chest radiograph continues to be normal, CT of the lungs should be performed within 24 hours. High-resolution (HT-CT) techniques are superior to spiral-CT scans in detection of any pathological findings. Although recently thin-section multislice CT is more in use in many institutions as it combines the potential of both techniques. If pulmonary infiltrates are confirmed by CT, a bronchoscopy with BAL should follow.[83]

For pulmonary infiltrates which cannot be identified by imaging techniques, fibre optic bronchoscopy with BAL and microbiology and non-culture based techniques, and which do not respond to antimicrobial therapy, trans-bronchial biopsy or open-lung biopsy should be considered. Patients with undiagnosed lung infiltrates do require histological identification (e.g. suspected invasive aspergillosis or non-infectious lung infiltrates). The presence of thrombocytopenia calls for open lung biopsy; whereas platelet counts greater than 50,000/L, a transbronchial biopsy may be performed, but if aspergilloma is suspected then transbronchial biopsy is contraindicated. Data from the literature show a complication rate of 10 to 15% after open lung biopsy.[83] Histological diagnosis of invasive aspergillosis from lung tissue obtained by biopsy or autopsy, is considered a "gold standard".[83]

1.4.4.5. Viral infections

Viral infections in patients with the diagnosis of haematological malignancy (leukaemia and lymphoma) are associated more with cellular immunosuppression than with neutropenia. Primary infections, reinfection and reactivations can occur. The most prevalent viral pathogens are herpesviruses (HSV, VZV, CMV, HHV6), particularly Cytomegalovirus (CMV) which can produce severe infections with high morbidity and mortality, such as hepatitis, pneumonia and gastrointestinal tract involvement. Adenoviruses, respiratory syncytial virus (RSV), as well as HHV6 are benign viruses normally, but in the immunocompromised host can be a cause of significant respiratory infections. Other viruses with documented infections in neutropenic patients are; influenza and parainfluenza viruses.[67, 86]

1.4.5. Prophylaxis

Intravascular Catheter-Related Infections: the recommendations designed to reduce the infectious complications associated with intravascular use are as follows; (1) educating and training health care providers who insert and maintain catheters, (2) using maximal sterile barrier precautions during central venous catheter insertion and use, (3) using a 2% chlorhexidine preparation for skin antisepsis, (4) avoiding routine replacement of Central venous catheters (CVCs) as a strategy to prevent infection, and (5) using antiseptic/antibiotic-impregnated short term CVCs if the rate of infection is high despite adherence to other strategies.[87]

 Studies on chemoprophylaxis with Quinolone has proven to reduce the frequency of documented Gram-negative infections, but occasionally may lead to an increase in Gram-positive infections.[67] Trimethoprim-sulfamethoxazole (TMP-SMZ) has shown to be highly effective in the prevention of pneumonitis due to *Pneumocystis carinii*.[88] Fluconazole reduces the frequency of both superficial and systemic infections with limited efficacy against *Candida krusei*, some strains of *C. glabrata* and molds. While Itraconazole has proven more effective than Fluconazole for long time prophylaxis of invasive fungal infections, with significant reduction in the

frequency of systemic fungal infections due to *Candida spp.*, and a decrease in candidiasis-related mortality.[88]

AmBisome 3mg/kg/day three times per week may be given as an alternative in patients with previously documented fungal infection or in patients required voriconazole as treatment (see appendix-4, A.4.1.). Most patients with haematological malignancy have already been exposed to viral infections, which undergo latency and reactivated with immunosuppression, Aciclovir is frequently given prophylactically.[88]

1.4.6.   Granulocyte colony stimulating factors

Hematopoietic colony-stimulating factors (CSFs), such as granulocyte CSF (G-CSF) and granulocyte–macrophage (GM-CSF), may be administered along with chemotherapy for their properties as to promote proliferation, differentiation and function of progenitor and mature cells of myeloid lineage. As well as stimulating the bactericidal function of mature neutrophils.[89]

Clinical trials have shown CSFs to shorten the neutropenic period and reduce incidence of febrile neutropenia in high risk patients by 50%. Other studies evaluating the role of CSFs administration in the treatment of febrile neutropenia have shown a reduction in the incidence of episodes of prolonged neutropenia or hospitalization. Subset analysis in two of the trials showed the greatest benefit in patients with profound neutropenia (ANC <100/mm3) and/ or documented infection.[89] Patients at higher risk of delayed haematological recovery or with severe infection are the most likely to benefit from the therapeutic use of CSFs as suggested by these studies.[89]

1.4.7. Management

Different institutions apply different policies for febrile neutropenic management, the following policy is followed by the Royal free hospital (RFH) in which this study took place (for diagnosis and management RFH policies see appendix-4).

Figure 1.2. Diagnosis and management of febrile neutropenic episode (modified; see appendix-4, A.4.1.).
Abbreviations: ANC; absolute neutrophil count, B/C; blood culture, CT; computed tomography, CVC; Central venous catheter, CxR; chest x-ray, HL; hickman line, hrs; hours, -ve; negative, +ve; positive, inf; infection R; treatment,
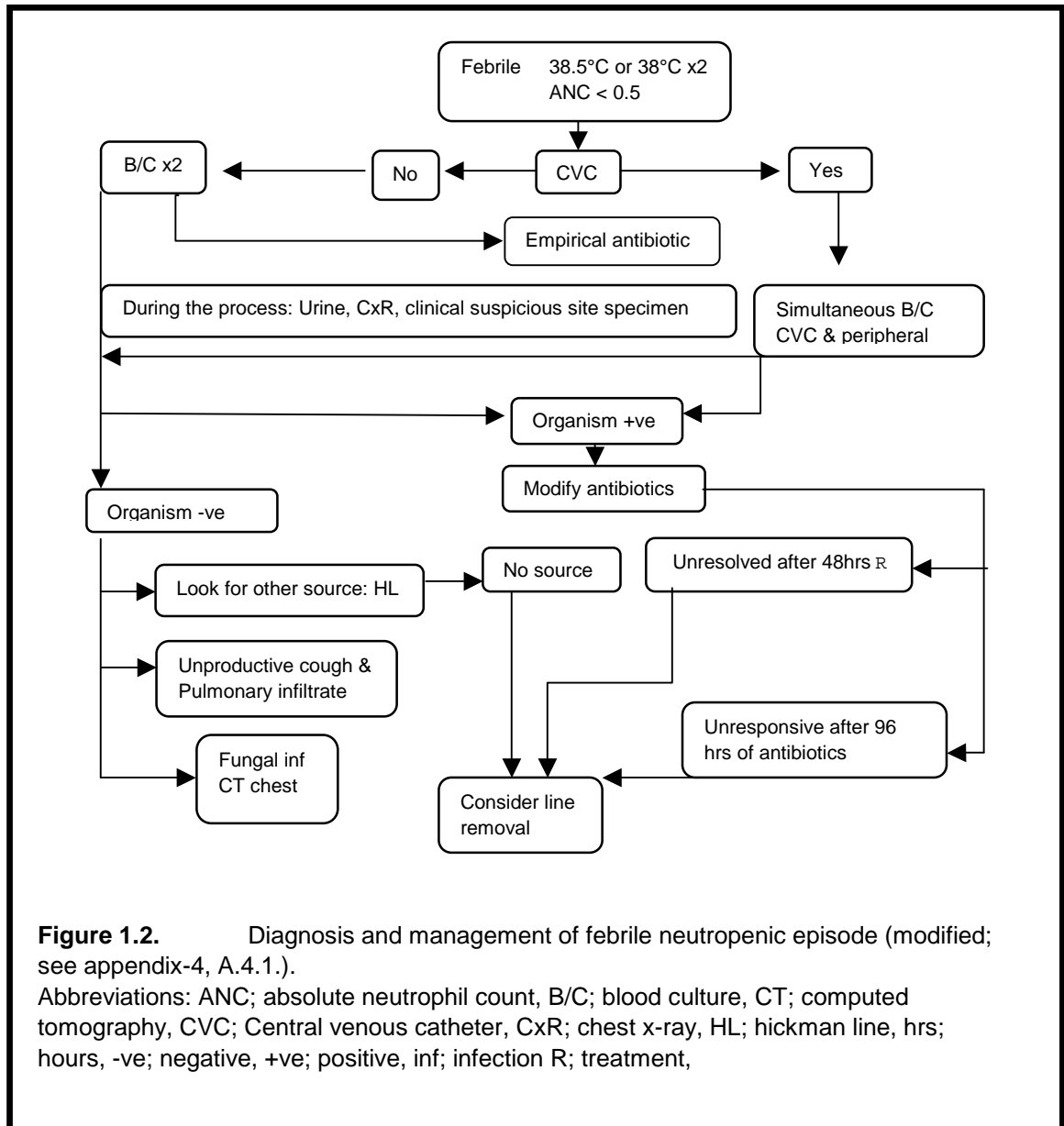
1.4.7.1.　　　Febrile neutropenia

When fever (>38.5°C or 38°C x2 (2hours apart)) occurs in the context of neutropenia (ANC <0.5 x10^9/l), empirical antibiotic therapy with β-lactam (meropenem) is started immediately after the collection of two blood cultures (B/Cs), drawn from two separate sites. Other investigations include urine, chest x-ray (CxR), and specimens from clinically suspicious sites. Blood cultures are repeated every 24 hours while the patient is febrile (see appendix-4, A.4.1.) and (figure 1.2.). Itraconazole level measurement is performed if patient is on itraconazole prophylaxis, and Galactomannan can be requested. Results can take up to three days to become available and this will be used to guide the empirical antifungal therapy after 96 hours (see appendix-4, A.4.1 & A.4.2.).

When a definite bacteraemia has been diagnosed (two or more sets of B/Cs positive for the same organism) antibiotic therapy is rationalized according to sensitivity (see appendix-4, A.4.1.). The duration of treatment with the same antibiotic does not exceed ten days. If fever is unresponsive after ten days, a change of antibiotic and discussion with a microbiologist is highly recommended. The patient should receive seven days course of the appropriate microbial sensitive antibiotic, with at least four days fever free (see appendix-4, A.4.1.).

The physician needs to consider the possibility of CVC removal, antibiotic cover, the type of antimicrobial therapy, according to organism sensitivity and the duration of antimicrobial therapy.[71] Infections caused by Coagulase-negative *Staphylococcus spp.* can be treated with glycopeptides antibiotics for 14-15 days, without catheter removal.[71]

Diarrhoea if proved to be due to *Cl. difficile* is treated orally by either metronidazole or vancomycin.[77] Standard therapy is for 10-14 days depends on symptoms,[3] (see appendix-4, A.4.1.). If the fever is unresponsive after ten days, a change of antibiotic and discussion with a microbiologist is highly recommended. Patients to receive seven days course of the appropriate microbial sensitive antibiotic, with at least four days fever free. In case of persistent fever despite 96 hours

administration of appropriate antibiotics, then other causes are considered. Most likely causes are fungal infection. The presence of non productive cough and the association with pulmonary infiltrates, necessitate an early BAL arrangement, and a HR chest CT (see appendix-4, A.4.1.).

If B/C has grown *Candida*, the patient should have an upper abdominal high resolution CT scan and an ophthalmological examination of their fundi (for the detection of choroidoretinitis / endophthalmitis), (see appendix-4, A.4.2.). If fever has responded to empirical antifungal treatment, this is continued till neutrophil recovery (ANC ≥ 0.5 x10^9/l),(see appendix-4, A.4.1.).

The azoles (fluconazole, itraconazole, variconazole and posaconazole) has became standard therapy for invasive candidiasis.[90] While the echinocandins (caspofungin, micafungin and anidulafungin) are fungicidal against *C. Albicans* and *non-albicans*[90] and they are considered along with fluconazole as effectious and saver alternative to the polyenes; amphotericin B.[71, 91]

Febrile neutropenia with lung infiltrate are treated with β-lactam antibiotics. While *pneumocystis carinii* pneumonia respond to treatment with TMP/SMX (Trimethoprim-sulfamethoxazole).[83] In presumed CRI (bacteraemia or fungaemia) antibiotic treatment is modified according to isolates continues for at least 10-14 days or longer if patient is neutropenic, and in case of tunnel and exit site infection until infection is resolved. Catheter removal should be considered if cultures remain positive after 48 hours of therapy or if proven infection with fungi, *staphalococcus aureus, bacillus spp., pseudomonas* or *mycobacterium spp*. In the absence of a source of infection, Hickman line removal should be considered. Other infections such as viral infections should always be first investigates appropriately, and managed accordingly if proven present, (see appendix-4, A.4.1.). Uncommon pathogens such as *corynbacterium spp* are treated with vancomycin, carbapenem or TMP-SMZ, while *mycobacterium spp* susceptibility varies by species.[91]

With severe life-threatening infections, focal lesions unresponsive to appropriate antibiotic therapy, or expected prolong neutropenia and delayed haematological

recovery; granulocyte transfusions may be useful and beneficial in those patients for its stimulating effect on the depleted neutrophils[89] (see appendix-4, A.4.1.).

1.4.7.2. Febrile non-neutropenia

Patients with febrile non-neutropenia should be investigated and treated as any other. Their infections and management do differ from neutropenic patients. Fever is investigated and confirmed by two sets of B/Cs. Treatment follows the empirical guidelines and is modified when an organism is identified. Levels of itraconazole are measured if patient is on itraconazole prophylaxis. Catheter related, exit site or tunnel infections management is the same as neutropenic patients (appendix-4, A.4.1.).

1.5. The role of genes in innate immunity

The immune system consists of two closely related and yet different responses-innate and adaptive immunity.[92] Mammalian innate immune recognition occur through toll-like receptors (TLRs) and NOD2 proteins,[93] which allows the phagocytes to recognize and react to a component of bacterial peptidoglycan (PGN) and muramyldipeptide (MDP).[94] Several lines of evidence have led to the hypothesis that impaired immunity initiates the cascade of events resulting in Crohn's disease (CD).[95]

Chitotriosidase is another component of the innate immune system, recently implicated in anti-fungal responses.[7] Involvement of chitotriosidase in the anti-pathogenic responses are suggested as activity of this enzyme was raised in neonates with fungal, bacterial and viral infections.[6] Other evidence was also seen in children undergoing treatment for AML, as children with mutant gene allele for chitotriosidase showed higher risk for gram negative infections.[96] These pathogens can be sensed by NOD2 and TLR. This finding was the basis of a study which

showed that chitotriosidase can contribute to TLR and MDP-dependent innate immune response required for eradication of protozoan parasites, nematodes, fungi and bacteria.[97]

The main aim of our study was to explore the Chitotriosidase and NOD2 genes and to look into the extent of it influence on innate immune response, in their wild type and mutant status and also in neutropenic and non-neutropenic patients undergoing chemotherapy for haematological malignancy.

1.6. Chitotriosidase

1.6.1. Identification & Expression

Chitin is a glycopolymer present as a structural component of many species such as parasitic nematodes, microfilarial sheat, fungi cell walls, insects, gut lining and the exo-skeleton of all types of arthropods.[98] Gaucher disease is an inherited metabolic disorder that exhibits marked elevation of activity of chitotriosidase in their plasma and hence the identification of a human phagocyte specific chitinase named chitotriosidase[98, 99] with the capacity to hydrolyze artificial chitotriosidase substrates.[100] Chitotriosidase can be synthesised in large amounts by tissue macrophages under an appropriate stimulus.[98] It is largely secreted as a 50-kDa (molecular mass) active enzyme containing a C-terminal chitin binding domain. In macrophages some enzyme is converted by carboxyl-terminal proteolytic processing into the C-terminal truncated 39-kDa isoform with hydrolase activity that accumulates in lysosomes of these cells. The 50 kDa chitotrisidase isoform is also synthesized by progenitors of neutrophilic granulocytes and stored in their specific granules.[98, 99] The C-terminal domain of 50-kDa chitotriosidase mediates a strong binding to chitin, enabling it to cleave chitotriose and hydrolyze colloidal chitin to yield chitobiose, in distinction from 39-kDa isoform which lack this ability.[101] At least two distinct types of chitinase are expressed in humans. Chitotriosidase 1 (CHIT1) was the first identified. Only recently, a second chitinase called acid mammalian

chitinase (AMCase) has been identified in humans.[98] The expression pattern of both chitinases vary completely, CHIT1 is exclusively produced by phagocytes, and increases in conditons involve macrophages activation,[102] whereas AMCase is expressed in alveolar macrophages and in the gastrointestinal tract.[103]

Chitotriosidase enzyme belongs to the family 18 of glycosyl hydrolases. It is an endoglucosaminidase that cleaves and shows transglycosylation activity towards chitin, a polymer of N-acetyl-D-glucosamine[7] and shares significant sequence identity from various non-mammalian species like plants, bacteria, fungi, nematodes and insects.[7, 98] Recombinant chitotriosidase has been found to inhibit hyphal growth of chitin-containing fungi such as *Candida* and *Aspergillus species*. This ability along with its specific expression by phagocytes suggests a physiological role in defence against chitin-containing pathogens.[7, 98] Other findings indicate that chitotriosidase can play an important role during the immunological innate response and in other diseases in which the inflammatory processes may be a key element.[7, 104, 105]

1.6.2.   Structure

The Chit gene is located on chromosome 1p13,[103] whereas the locus of the crystal structured[101] human chitotriosidase gene ois found on chromosome 1q32.[103] The gene consists of 12 exons and spans about 20 kb of genomic DNA. Exon 11 can be alternatively spliced. This exon is usually skipped in the splicing process generating the predominant mRNA form encoding the 50 kDa protein. In macrophages, a different form of mRNA is rarely produced as a result of a lack of exon 11 skipping (39 kDa)[99, 106] (figure 1.3.).

Other inactive members of the mammalian chitinase protein family in human have been identified: human cartilage glycoprotein 39 (HCgp-39/ YKL40), YKL39, and oviductin (specific glycoprotein). Although other homologies were observed between the four human chitinases, chitotriosidase was the only one observed with glycosyl hydrolase activity, and the lack of catalytic acidic amino acid residues. The

speculations that this family may play a role in chemotaxis and tissue remodelling is yet to be confirmed.[98]

AMCase is another mammalian chitinase. It is characterized by an acidic pH optimum and extreme stability at acid pH. AMCase is relatively abundant in the gastrointestinal tract (stomach) supporting a possible role as providing a physical barrier and protection against pathogens and food particles as well as facilitating digestion. Its presence was also observed in the lung but to lesser extent with the possibility of the involvement in lung inflammation.[98] AMCase and the human macrophages chitinase, do not only share the same 18 glycosyl hydrolases, the N-terminal catalytic core domain of 39kDa and a C-terminal chitin binding domain, but also 52% sequence identity and the additional α/β folds. Given the differences in expression and the stability of the additional mammalian chitinase at pH of around 2, it is likely that AMCase compared with its human analogue chitotriosidase has a different role.[98]
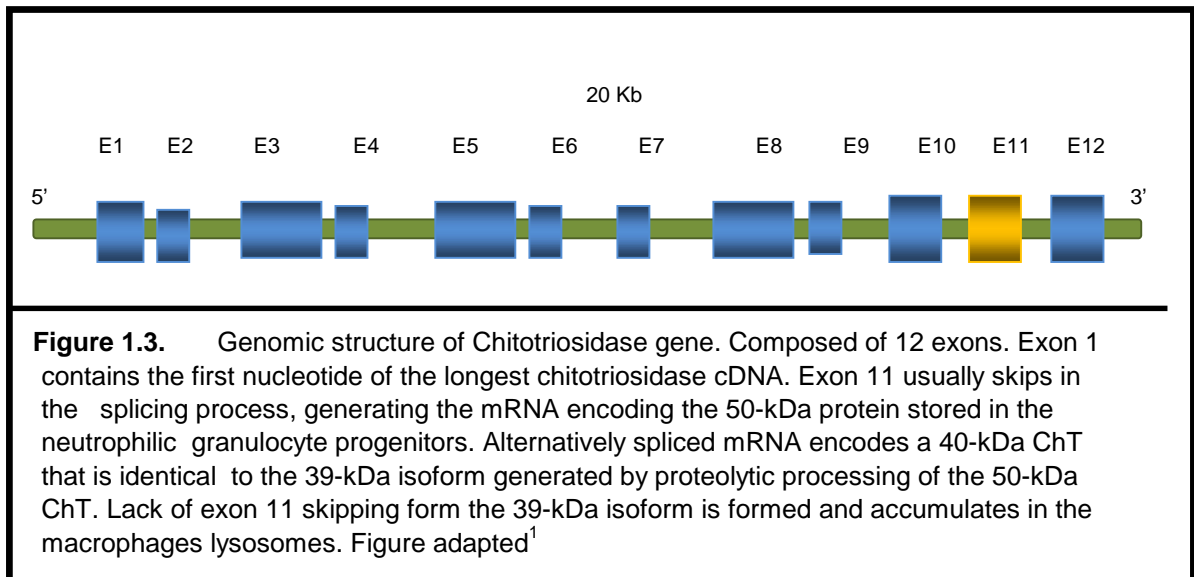
Figure 1.3. Genomic structure of Chitotriosidase gene. Composed of 12 exons. Exon 1 contains the first nucleotide of the longest chitotriosidase cDNA. Exon 11 usually skips in the splicing process, generating the mRNA encoding the 50-kDa protein stored in the neutrophilic granulocyte progenitors. Alternatively spliced mRNA encodes a 40-kDa ChT that is identical to the 39-kDa isoform generated by proteolytic processing of the 50-kDa ChT. Lack of exon 11 skipping form the 39-kDa isoform is formed and accumulates in the macrophages lysosomes. Figure adapted[1]

Figure 1.4. The differences between the normal and the mutant chitotriosidase gene. Exon 10 contains 24-bp duplicate in the mutant gene. Figure adapted[1]
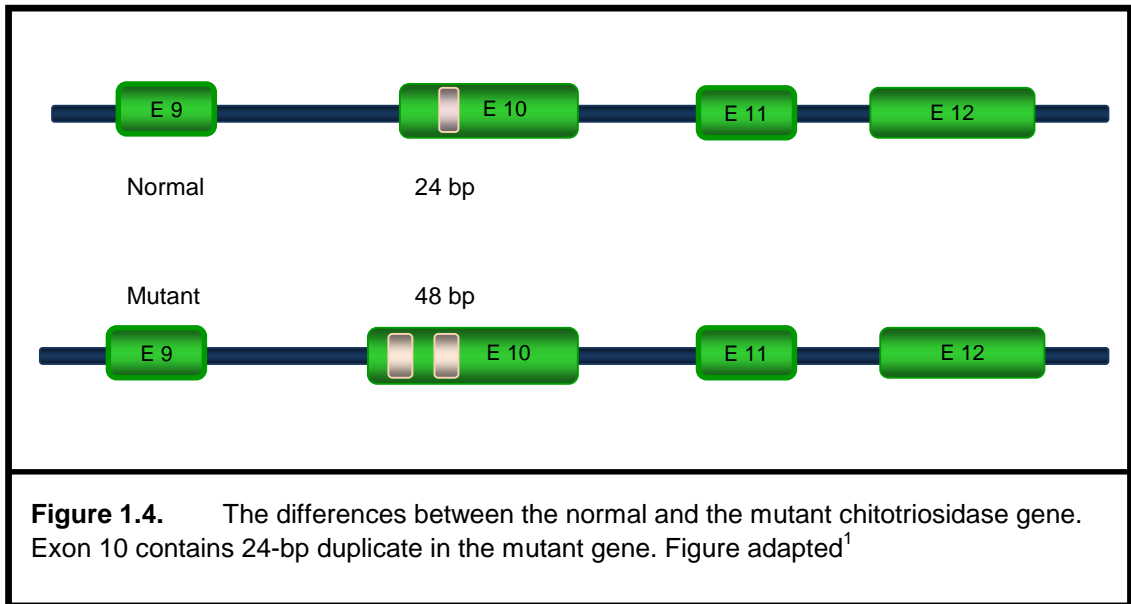
1.6.3. Polymorphism

Deficiency in chitotriosidase activity is seen in different ethnic groups. Inheritance is frequently recessive. The defect was found to be 24-bp duplication in exon 10 that activates a cryptic 3' splice site in the same exon, causing the formation of an abnormally spliced mRNA with an in-frame internal deletion of 87 nucleotides (figure 1.4.). The spliced mRNA encodes an enzymatically inactive protein that lacks an internal stretch of 29 amino acids.[106] Incidence of the mutation in chitotriosidase allele is 37% present in Ashkenazi Jews and Dutch individuals, whereas both populations were about 6% homozygous for this allele.[106] Studies performed in a Portuguese population showed approximately 40% of the general population are heterozygous, while 6% were homozygous for the chitotriosidase activity deficiency.[107] Recently two other polymorphisms in chitotriosidase beside 24bp duplications were identified; G354R and A442V. These two polymorphisms were found predominantly in African descent individuals, and is associated with reduced chitotriosidase activity.[108] Chitotriosidase reduced activity is also found in

3–5% of individuals in the general population due to the presence of a null allele in the chitotriosidase gene.[106]

The multi-ethnic occurrence and prevalence of chitotriosidase gene mutation suggests that the enzyme is redundant in man and that this mutation is relatively ancient in evolution. Chitotriosidase deficient individuals showed no chitotriosidase in all materials tested including plasma, urine, leukocytes, and tissues, indicating that this mutation is the predominant cause of chitotriosidase deficiency.[106]

According to the absent enzyme activity in the homozygote individuals for the mutant allele and the approximate half activity in the heterozygous, it appears that chitotriosidase deficiency in humans is autosomal incompletely dominant disorder. Macrophages from chitotriosidase deficient individuals when cultured contain very little mRNA and secrete almost no chitotriosidase protein.[1]

1.6.4. Chitotriosidase function & disease associations

1.6.4.1. Chitotriosidase and immunity

Tissue macrophages are placed in almost all organs, protecting the microenvironment and are considered an important element in the host defence. Under pathological or physical conditions macrophages are capable of synthesizing and producing large amount of chitotriosidase.[98] It represents approximately 1% of the total protein secreted by the macrophages.[99]

In order to elicit the cellular response and observe the activity level of chitotriosidase, a study based on the increased cytocidal activity against intracellular microorganism in the IFN-γ and TNF-α treated macrophages was carried out. In this study macrophages treated with IFN-γ, TNF-α and LPS showed upregulation of chitotriosidase gene expression.[109]

Evidence of enhanced chitotriosidase activity was also demonstrated after IL-12 injection in chimpanzee.[110] Since IL-12 is a potent immune regulatory cytokine that

is crucially involved in a wide range of infectious diseases (viral, bacterial and intracellular parasitic infestation),[111] it is reasonable that the elevated levels of IL-12 modulate the macrophage activity, stimulating their microbicidal function through a pathway involving chitotriosidase activity.[112] This idea can be supported by the studies showing that in malaria protective immunity is mediated by a cascade of events involving IL-12[113] and the raised chitotriosidase level in African children effected with malaria (42%) compared to the control in the same group (12.24%)[112] (table1.2.). In addition, prolactin which is structurally related to cytokines had also shown the ability to up-regulate CHIT1 mRNA expression in human macrophages.[114]

Studies have revealed the activity of chitotriosidase against chitin-containing pathogens. It was found to inhibit the growth of Cryptococcus neoformans by causing hyphal tip lysis in *Mucor rouxii*. In *Candida albicans* it prevented the occurrence of hyphal switch by exhibiting the chitinolytic activity towards it's cell wall chitin and colloidal chitin.[7, 98] Furthermore, increased chitotriosidase activity was found in the plasma and urine in neonates with systemic fungal infections[6] (table 1.2.). GM-CSF treated macrophages, showed increased chitotriosidase transcripts, and beneficial effects in fungal infections, promoting chitotriosidase release from Polymorphnuclear neutrophils (PMNs) through exocytosis of specific granules.[7]

Studies by Eijk *et al*[7] on chitotriosidase deficiency showed improved survival in a neutropenic mouse model of systemic Candidiasis and Aspergillosis after use of recombinant chitotriosidase. These findings suggest a possibility of treating life threatening fungal infections with recombinant chitotriosidase in mutant allele carriers.[7] The other study in chitotriosidase deficient patients was in immunocompromised patients due to chemotherapy, and the increase susceptibility to gram negative bacterial infections.[96] Chitotriosidase deficiency has been linked to increase susceptibility to parasitic infections, as seen in the high incidence of human filariasis due to *Wuchereria bancrofti* in deficient patients[115] (table 1.3.).

As seen from the above studies chitotriosidase released from either the specific granules in neutrophils or activated macrophages, is involved in innate immunity and the immunological response required for the eradication of protozoan, nematodes, fungi and bacteria.[7, 97] Other members of chitinase family are also likely to be involved in the immune response.

1.6.4.2. Chitotriosidase and Gaucher Disease

Gaucher disease (GD) is an autosomal recessive lysosomal disorder. It is due to deficient activity in the lysosomal enzyme, β-glucosidase (glucocerebrosidase), a lysosomal hydrolase. Deficiency of this enzyme leads to the accumulation of glucocerebrosides in the lysosomes of macrophages, resulting in lipid-laden macrophages referred as Gaucher's cells.[100, 116] Gaucher disease is the most common lysosomal storage disorder with high prevalence among Ashkenazi Jews, 1/885 compared to 1/100,000 in other populations.[116] There are three clinical types of GD. The most common, is the chronic non-neuropathic type-1 form of the disease, which shows highly variable signs, symptoms and course. Presentation could be due to visceral, haematological or skeletal involvement.[100, 116] Accumulation of lipid-laden macrophages (Gaucher's cells) in the affected individuals causes the development of hepatosplenomegaly, bone lesions and, occasionally, neurological abnormalities. Patients with GD need regular assessment of the biochemical variables, as abnormalities reflect the extent of bone or visceral involvement. Some biochemical variables have been found to be of value in monitoring both the course of the disease and the response to treatment.[100] The most prominent biochemical abnormality is the marked elevation of plasma chitotriosidase activity, and it was first observed by Hollak and co-workers in the plasma samples of untreated type-1 GD.[100] In the same study there was a slight elevation in chitotriosidase activity even in asymptomatic glucocerebrosidase-deficient individuals while Alkaline phosphatase (AP) was well within the control range[100] (table 1.2.).

Enzyme-replacement therapy has proven to be effective in improving the clinical, haematological and biochemical (plasma markers) parameter of GD patients. Plasma chitotriosidase activity is a useful biomarker of macrophages activity (phagocytic activity of lipid laden macrophages) for monitoring GD patients for its correlation with disease severity, activity and its sensitivity to treatment.[117]

More modest elevations in plasma chitotriosidase have been noted in some other inherited lysosomal storage disorders, especially sphingolipidoses such as Niemann Pick, GM1-gangliosidosis, and Krabbe's disease[118] (table 1.2.).

1.6.4.3.   Chitotriosidase & bronchial asthma

Since the identification of the second chitotriosidase-AMCase and its expression in the alveolar macrophages[98] have been in progress to see if inflammation in the lung and pathogensis of bronchial asthma is associated with this finding. Recently an association of AMCase polymorphisms and haplotypes with paediatric bronchial asthma was observed[119] (table 1.2.). This was followed by another study of the amino acid variants Gly 102Ser and Ala 442Gly, as well as the 24 bp duplication within CHIT1 in the pathology of bronchial asthma, which showed no such association in Caucasian children.[120]

Chitotriosidase activity was found to be significantly higher in active sarcoidosis than inactive form of the disease. This finding could indicate that chitotriosidase may be a useful marker in measuring and monitoring disease activity.[121]

1.6.4.4.   Chitotriosidase & thalassemia

Beta thalassemia patients need regular blood transfusions leading to iron overload and an enormous expansion of the reticulo-endothelial system. High plasma levels of chitotriosidase, comparable to the levels found in GD patients, were found in 10% of patients with β-thalassemia major. Level of chitotriosidase varies with the

type of thalassemia and the degree of requirement for transfusion. Modest elevation of chitotriosidase was found in the patients with β-thalassemia intermedia. The increase of plasma chitotriosidase activity in β-thalassemia major patients could be related to iron-mediated damage to the lysosomal apparatus. In β-thalassemia the amount of abnormal transfused red blood cells exceeds the macrophages degrading capacity. As a consequence, phagocytes of β-thalassemia patients become laden with membranous lipid material, macrophages activation and increased chitotriosidase production[122] (table 1.2.).

1.6.4.5.   Chitotriosidase & Coronary artery disease

Atherosclerosis is an inflammatory disease that is characterized by progressive deposition of lipids in the arterial wall. Pathogenesis involves endothelial cells activation and monocytes infiltration of the vessel wall. The monocytes differentiate into macrophages and accumulate lipids from the circulation and form the so called foam cells.[123] Recently studies showed a clear connection between chitotriosidase activity and lipid-laden macrophages inside human atherosclerotic vessel wall reflecting its severity and extend. It was more prominent in the atherothrombotic, and stroke group.[124] An increase in the serum chitotriosidase activity was found to be age depended. This could be explained by the gradual progression of atherosclerotic process.[123] There was no association between plasma chitotriosidase level and plasma lipid level before and after lipid lowering treatment. These suggest that inflammation related chitotriosidase activity is associated with the atherosclerotic process and extent of coronary artery disease[123] (table 1.2.).

1.6.4.6.   Chitotriosidase & Neurodegenerative Disorders

The heterogeneous nature of both Alzheimer's disease (AD) and stroke and the inflammatory aspects of the pathogenesis of these diseases are prominent. Inflammatory pathogenesis is mainly based on the association of the presence of

activated immune cells necessary for an immune response and the finding of raised levels of pro-inflammatory cytokines.[105]

Alzheimer's disease (AD) is a progressive neurodegenerative disorder showed a significant increase in chitotriosidase level, as an expression of immune activation in AD patients and to the ischemic injury in stroke patients.[105] In stroke the increase clearly correlates with the clinical-radiological severity of the stroke. Chitotriosidase is a specific indicator of the macrophage activation which may be of importance for future therapeutic intervention in stroke aimed at the immune response in these events[125] (table1.2.).

Sotgiu *et al*[126] in his study showed that macrophage-derived enzyme chitotriosidase is significantly elevated in multiple sclerosis (MS), and the plasma and cerebrospinal fluid level parallels with the clinical MS symptoms and deterioration. In addition, intrathecal chitotriosidase activity better correlates with the extent of CNS damage than macrophage-derived markers.[126] Together, these findings add to the evidence of a local, self-sustaining immune and inflammatory response within cerebrovascular disorders[105] (table 1.2.).

1.6.4.7.   Chitotriosidase & non-alcoholic steatohepatitis

The initiating event in NASH (non-alcoholic steatohepatitis) seems to involve early lipid accumulation and lipid peroxidation in the hepatocytes, followed by Kupffer and HSC (human hepatic stellate cell) activation, liver cell injury, inflammation, and eventually hepatic fibrosis. Kupffer cells are a source of proinflammatory cytokines, and the only source of chitotriosidase expression in liver. In fact, the highest levels of chitotriosidase was observed in patients with the highest degree of hepatic iron accumulation, as confirmed by the high correlation observed between chitotriosidase and ferritin levels in both Kupffer cell lysates and plasma of NASH patients. It appears that the increase of chitotriosidase in Kupffer cells may reflect iron mediated damage to lysosomes, suggesting that the physiological function of chitotriosidase probably lies in regulating the acute phase response, and that it is

not only a biochemical marker of macrophage activation but can also be regarded as an important player in the inflammatory process[104] (table 1.2.).

Table 1.2. Studies relating chitotriosidase activity in diseases in patients with wild type allele

Disease	Reference	No of patients	Increased baseline activity
Gaucher disease	Hollak[100]	32	Decline with treatment
Fabry disease	Vedder[127]	29 males 38 females	Minor elevation Not
Krabbe disease	Wajner[118]	9	Plasma
GM1-gangliosidosis	Wajner[118]	19	Plasma
Sarcoidosis	Brunner[121]		With disease activity
Fungal infections	Labadaridis[6] Masoud[128]	8 55	Plasma & urine No association
Bacterial infections	Labadaridis[6]	15	Plasma & urine
Malaria falciparum	Barone[112]	67	Plasma
Thalassemia	Barone[122]	72	Plasma
Atherosclerosis	Artieda[124]	153	Plasma
Coronary artery disease	Karaday[123]	200	Plasma
Strokes	Sotgiu[125]	44	Correlate with severity
Alzheimer's disease Cerebrovascular dementia	Di Rosa[105]	40 40	Increase expression, and enzyme activity
Multiple sclerosis	Sotgiu[126]	77	Correlate to severity
NASH Simple steatosis	Malagurnera[104]	40 20	Markedly increase Mod. elevation
Asthma	Bierbaum[119]	322	AMCase involvement in pathophysiology

Table 1.3. Increase susceptibility of infections with chitotriosidase mutant gene.

Disease	Reference	No of patients	Decrease activity
W. bacrofti	Choi[115]	216	Correlate with susceptibility

1.7. NOD2 gene

In 1996 (using genetic linkage studies), a Crohn's disease-susceptibility gene locus on chromosome 16q12 was identified. Named IBD1 (inflammatory bowel disease locus 1) it lies in the pericentrometric region. It may be inherited in a recessive manner, and is specially associated with Crohn's disease (CD).[129] The NOD2 (nucleotide oligomerization domain) gene within the IBD1 locus was discovered by two groups in 2001 (Hugot *et al*[130], & Ogura *et al*[4]), who showed that mutations of the NOD2 gene were strongly associated with CD.[4, 130] Hugot *et al*[130] screened 235 CD families to identify the NOD2 gene on the IBD1 locus and the three polymorphisms associated with CD using a positional-cloning strategy, based on linkage analysis followed by linkage disequilibrium mapping[130] (figure 1.5.).

Ogura *et al*,[4] identified the NOD2 whilst investigating apoptotic proteins, and intuitively considered NOD2 a candidate gene on the IBD1 locus as this gene is located in the peak region of linkage on chromosome 16 that is associated with CD.[4]

1.7.1. NOD2 Protein

1.7.1.1. The structure of NOD2 protein

The NOD2 gene encodes a protein of 1040 amino acids. It is composed of two N-terminal caspase recruitment domains (CARD) (residues 28-220), fused to centrally located nucleotide-binding domain (NBD) (residues 273-577) containing consensus nucleotide-binding motifs, followed by 10 tandem leucine-rich repeats (LRRs) (residues 744-1020)[131] (figure 1.5.).

The NOD2 protein is a member of a large family of intracellular proteins, the NOD proteins. They are distrributed widely in nature, so far over 20 NOD containing proteins have been identified in humans (figure 1.6.), plants and nematodes. The disease resistance (R) proteins seen in plants are involved in plant host defence

against pathogens.[131] The family also include NOD1. Both NOD1 and NOD2 are close relatives and have similar structures except for the number of CARDS (figure 1.6.), as NOD2 is the first protein to contain two CARDs.[131] The majority of animal and plant NOD-LRR proteins are comprised of three distinct functional domains: CARD; an amino-terminal effector domain involved in signalling, NBD; a centrally located regulatory NOD domain, and carboxyl-terminal LRRs that serve as a ligand-recognition domain (figure 1.5.).[132] Alternatively the NOD-LRR proteins are also known as the CATERPILLER (CLR) an acronym for CARD, transcription enhancer, R (purine)-binding, pyrin, lots of leucine repeats. A family that have been implicated in intracellular recognition of bacterial components.[132]

1.7.1.2.   CARD domain

The caspase recruitment domain (CARD) is a protein fold consisting of a six membered α-helical bundle. The CARD domain facilitates caspase aggregation and activation at the onset of apoptosis.[133] These effector domains are involved in homophilic interactions with downstream signalling molecules.[132] Proteins containing CARDs, or related motifs, are centrally involved in assembling protein complexes that drive activation of either caspases or IκB kinase (IKK) by facilitating close proximity of their molecules, linking these proteins to multiple signalling pathways and biological functions. The CARDs are responsible for oligomerization with both self and other proteins. It is physically associated with RICK (serine/threonine kinase), through homophilic CARD-CARD interactions.[132-134] RICK bind to the IKK complex through IKKγ. This interaction induce phosphorylation of IKKα and/or IKKβ subunit, which subsequently results in nuclear factor kappa B (NF-κB) activation.[135]
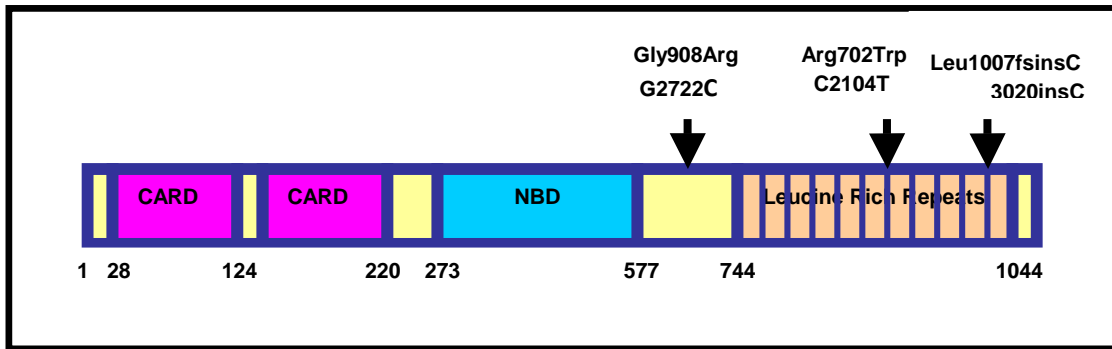
Figure 1.5. Domain structure of NOD2. Illustrating the site of protein truncation; showing   CARD, NBD, and ten LRRs. Numbers indicate residue positions. Figure adapted.[4, 130]
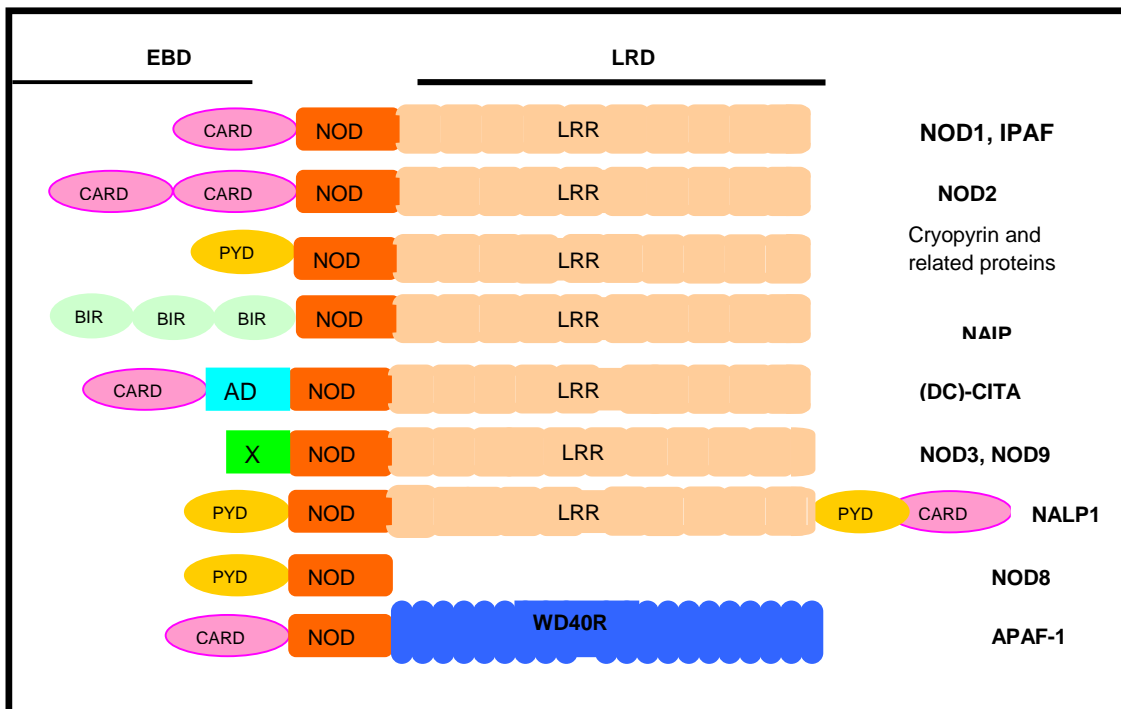
Figure 1.6. The domain structure of the NOD proteins in human. Most NOD proteins are composed of variable amino-terminal effector-binding domains (EBD), a centrally located nucleotide-binding oligomerization domain (NOD) that mediates self-oligomerization, and a carboxyl-terminal ligand-recognition domain (LRD). The number of leucine- rich repeats (LRRs) varies in NOD proteins. Other abbreviations: PYD; pyrin domain,BIR; baculoviral inhibitor-of-apoptosis repeat,APAF-1; apoptotic protease activity factor 1. Figure adapted.[132]

1.7.1.3.　　NOD domain

The centrally located nucleotide oligomerization domain (NOD) of NOD2 mediates self-oligomerization leading to NF-κB activation.[134] The NOD domain contains structural motifs found in a class of nucleoside triphosphatases (NTPases) referred to as the ATP-binding cassette (ABC) proteins. The ABC region in all NOD proteins includes catalytic residues with binding site motifs which are essential for binding phosphate residues and magnesium ions. They are also predicted to be involved in hydrolysis of nucleotides, ATP, deoxyATP (dATP) and/or GTP.[132, 136]

1.7.1.4.　　LRR (leucine-rich repeat) domain

The plasma membrane toll like receptors (TLR) play an important role in the recognition of pathogen-associated molecular patterns (PAMPs) and mediating innate immunity activation.[137] Plant disease-resistant (R) proteins have C-terminal LRRs that are similar to toll like receptor-leucine rich repeat (TLR-LRR) and are essential for defense and pathogen recognition. These cytosolic proteins recognise distinct effector molecules of wide array pathogens including bacteria, fungi and viruses. It is currently suggested that the NOD2-LRR perform a similar function.[131, 138] Each of the 10 LRRs of NOD2 encode predicted α helix and β sheet sequences that are consistent with the prototypical horseshoe-shaped structure of R protein LRRs. The functional role for the LRRs of NOD1 and NOD2 remains unclear.[131]

1.7.2.　　NOD2 protein Function and role in immunity

The NODs and plant R proteins structural similarity led to functional studies that suggest the NOD2 function is as an intracellular receptor to bacterial lipopolysaccharide (LPS) and peptidoglycan (PGN). Furthermore, they suggest that the LRR motifs are not only essential for the recognition, but also for the response to LPS and muramyl dipeptide (MDP); (a basic PGN motif that is common to both gram positive and gram negative bacteria and is known to induce pro-inflammatory

cytokines), by NOD2 and the subsequent initiation of a cascade that activates NF-κB signalling (figure 1.1.). These findings were consistent with the evidence that LPS interacts with the LRR domains of R proteins and TLR with subsequent NF-κB activation.[93, 138, 139]

NF-κB stimulation results in nuclear translocation of the nuclear factor and genes which include TNFα and interleukin-12 (IL-12), pro-inflammatory cytokines which are seen in abundance in CD. NF-κB binding sites are also found on the NOD2 promoter region and studies have shown that the cytokines TNFα and IFNγ can up-regulate NOD2 expression.[140]

The extracellular TLRs can recognise specific PAMPs, and so is their involvement in the innate immune response. The antigen-presenting cells (APCs) receptor, NOD2, and the CARD-containing RICK (serine/threonine kinase) transduces signals from receptors of both innate and adaptive immune system in response to PAMPs and the intracellular recognition of PGN motifs.[141, 142] These result in NOD1 and NOD2 olgmeraziation, which induce RICK, the serine/threonine kinase, recruitment via CARD-CARD interactions and NF-κB activation. NODs and RICK interact via their respective CARD domains, and induce recruitment of the IκB kinase (IKK) complex of IKKα, IKKβ, IKKγ to the central region of RICK and the sequestration of the NF-κB transcription factors inhibitory proteins; IKK complex. Active NF-κB is released and translocated to the nucleus inducing the transcription of various pro inflammatory cytokines, a result of the proteolytic degradation of IKK proteins and IκB phosphorylation.[94, 141-143]

1.7.3.   NOD2 Protein Expression

1.7.3.1.   Blood

Initially NOD2 expression was thought to be restricted to monocytes in comparison to NOD1 which is ubiquitously expressed in tissues.[131] But later studies observed

the expression in other blood leukocytes, namely granulocytes, dendritic cells and to lesser extent T lymphocytes, although not as abundantly as monocytes.[144]

1.7.3.2.   Intestine

1.7.3.2.1.   Epithelial Cells

Expression of NOD2 mRNA in the intestinal epithelial cells was soon investigated. Observations showed NOD2 mRNA and protein expression was upregulated by TNFα and γIFN in intestinal epithelial cells and in primary colonic epithelial cells.[140]

1.7.3.2.2.   Paneth Cells

Lala *et al* demonstrated NOD2 mRNA and protein expression in Paneth cells in the small intestine with the greatest intensity in the terminal ileum. The NOD2 expression was increased in patients with inflammatory bowel disease in the terminal ileum Paneth cells, and colon due to the presence of metaplastic Paneth cells.[145]

1.7.4.   NOD2 mutations

Using transmission disequilibrium test and case-control analysis, followed by pedigree disequilibrium[130] three single nucleotide polymorphisms (SNPs) were identified in the LRR domain and are association with CD: a frameshift mutation caused by cytosine insertion in exon 11 (SNP13) at nucleotide position 3020 (3020insC) resulting in a frameshift at the second nucleotide of codon 1007, and substitution of proline for leucine in the tenth LRR, followed by a premature stop codon. The two other SNPs (SNP8 & SNP12) in NOD2 are point mutations encoding amino acid substitutions. SNP 8 substituting Cytosine for Tryptophan at nucleotide position 2104. SNP 12 substituting Guanine for Cytosine at nucleotide position 2722. These two mutations, SNP8 & SNP 12, encode for amino acid substitutions, Tryptophan substituted for Arginine at codon 702 (Arg702Trp) and

Arganine substituted for Glycin at codon 908 (Gly908Arg). These three major mutations all occur within or near the LRR-encoding region of the gene. Over 30 other much rarer amino acid polymorphisms occurring near or within LRR-encoding region of the gene have also been described.[130, 132]

1.7.5. Disease mutation associations

1.7.5.1. NOD2 mutations and Crohn's disease

Crohn's disease (CD) is a chronic inflammatory disorder that affects any part of the gastrointestinal tract, from the oropharynx to the perianal area. It is characterized by alternating periods of active inflammation and remission. Due to the transmural inflammation complications such as abscesses, fissures, fibrosis, fistulas, strictures and bowel obstruction may occur. Crohn's disease susceptibility alleles, located in the third part of NOD2 gene, two missense, SNP8 the 2104C→T, and SNP12 the 2722G→C, and a frameshift 3020insC, SNP13, represent 82% of the mutant chromosomes in CD.[146]

In a comparative study of NOD2 allele frequencies in 3575 healthy Caucasian individuals from sixteen different centres from Europe, North America and Australia, significant heterogeneity between centres was found with Australia and Finland having the lowest frequencies of known variants while Belgium and Canada has the highest. NOD2 risk alleles are absent from Japanese and Korean populations and in Han Chinese. The pattern that emerges from these studies both of disease groups and healthy controls suggests that CD occurs at quite variable rates across geographical regions and that in some populations where NOD2 risk allele carriage rates are at a high, those alleles are present in up to 50% of individuals with CD. Nevertheless the incidence of CD in populations with substantial carriage of risk alleles, such as Belgium and Canada is remarkably similar to those with lower rates, suggesting that CD occurs in those groups because of other genetic susceptibilities.[147] Economou *et al* meta-analysis quantified distinct risk for CD for the three common NOD2 variants, and showed

increase odds to 2 fold with SNP8, 3 fold by SNP12, and 4 fold by SNP13 in non Jewish descent Caucasians.[148] The carriage of at least two heterozygote variants or homozygote mutation increased the risk to 20 fold,[148, 149] and in another study up to 40 fold.[146] The overall frequency allele in CD was 0.2% for SNP8, 0.3% for SNP12 and 0.4% for SNP13.[150]

Hampe *et al* estimated that about 18% of the genetic risk in the CD population can be attributed to this mutation,[151] while Economou *et al* found a modest impact of NOD2 mutations on familial CD,[148] and colonic CD.[149] There is a strong association with stenosing CD and ileal disease location.[145, 148] In adult population, association between NOD2 mutation and early-onset of disease has been observed and in children, NOD2 mutation confers an increased risk of surgery, fistulising and fibrostenosing disease behaviour have also been described[148, 152, 153] (table 1.4.).

1.7.5.2.   Blau Syndrome

Since the association between NOD2 mutations and CD was identified, other disease has been investigated in correlation with NOD2 mutation.[147]

Blau syndrome (BS) is a rare autosomal dominant disorder characterized by early onset granulomatous arthritis, uveitis and skin rash.[154] Linkage analysis has identified the susceptible locus for BS mapped to chromosome 16; NOD2. Blau syndrome susceptibility occurs due to mutations in the NBD of protein in contrast to CD in which mutations are in the LRR domain[155] (table 1.4.).

Three types of missense point mutations in the NOD2 have been discovered in BS families[147, 154, 155] NOD2 mutant proteins that are found in patients with BS are defective in their response to bacterial components and induce increased basal NF-κB activity. In contrast to CD associated NOD2 variants which have normal or reduced levels of basal activity.[93]

Table 1.4.   Studies performed in NOD2 to determine mutation and disease association

Disease	Reference	No of patients	Relative risk
Crohn's Disease	Hugot[130] Ogura[4] Economou[148]	235 families 56 families 42 studies	Risk rise; 2-4 folds in heterozugote & 20 folds in homozygote. Familial CD; slight risk (OR 1.49, 95%CI 1.18-1.87). Stenosing CD mod risk (OR 1.94, 95% CI 1.61-2.34). Small bowel CD (OR 2.53, 95% CI 2.01-3.16)
Blau Syndrome	Miceli-Richard[155]	4 families	Disease gene
Sarcoidosis	Kanazawa[154]	10 families	Association with early progressive form
Colorectal cancer	Kurzawski[156] Suchy[157] *Tuupanen[158]*	556 pt 607 pt 1042	Risk increase >50 yrs OR 2.2, CI 1.23-4.10 OR1.39, CI 0.91-2.14 *No association*
MALT risk in H pylori	Rosenstiel[159]	511 pt	OR 2.4, CI 1.2-4.6
Breast cancer	Lener[160] Huzarski[161]	4107 pt 462	Increased susceptibility (not quantitated) 5 fold risk <50 yr
NHL	Forrest[162]	904 pt	OR 3.1, 95% CI 1.1-8.8
Sepsis	Ahrens[163] Brenmoehl[164]	356 infant 132 pt	Borderline. OR 32, 95% CI 1.0-10.4 Increased mortality
GvHD	Holler[8] Mayor[9] Sairafi[10]	169 pt 196 pt 455 pt	TRM (both mutant); OR 6, 95% CI 2.6-14.1 OS; OR 1.617, CI 1.080-2.421. Decrease DFS; 1.603, 1.079-2.381. Increase relapse; 2.579, 1.538-4.323 *No association*
Psoriatic arthritis	Rahman[165]	187 pt	OR 2.97, CI 1.61-5.47
Allergic rhinitis Atopic dermatitis Increased IgE level	Kabesch[166]	1872	3 fold risk 2 fold risk
Coronary heart disease	EL Mokhtari[167]	900 pt	*No association*
Lung CA	Lener[160]	389 pt	*No association*
SLE	Ferreiros-Vidal[168]	189	*No association*
Behcet's disease	Ahmad[169]	374	*No association*
Multiple sclerosis	Sawcer[170]	631	*No association*
Ankylosing spondylitis	Van der Paardt[171]	113 pt	*No association*
Rheumatoid arthritis	Ferreiros-Vidal[172]	210 pt	*No association*
Pulmonary TB	Stockton[173]	320 pt	*No association*
Periodontitis	Laine[174]	104 pt	*No association*

1.7.5.3.　　Graft versus Host Disease

Studies have observed differences in the outcome of patients receiving stem cell transplant when the donor, recipient or both carries NOD2 mutation. Holler *et al* studied the outcome of gastrointestinal graft versus host disease (GvHD) and transplant-related mortality. At one year, they observed a significant increase in these parameters compared to wild type NOD2 patients. The overall incidence of GvHD increased when the donor carries the mutant gene with further dramatic increase when both donor and recipients are carriers of the mutation. As a consequence, cumulative incidence of one year transplant related mortality increased from 20% with wild type NOD2 in both donor/recipient, to 49% when the recipient is mutant for the allele, to 59% when the donor is mutation positive, and 83% if both donor and recipient revealed mutations.[8] Recently another study showed no direct effect on GvHD, although overall survival was decreased as well as disease free survival which was contributed to increase relapse incidence. The association of NOD2 mutations characterized by a diminished NF-κB response, however, with GvHD and transplant related mortality suggest a predominant role of an impaired monocyte/macrophage response resulting in dysregulation of inflammation and impaired microbial response of the gastrointestinal epithelium and possible sepsis[8, 9] (table 1.4.).

1.7.5.4.　　Sepsis

The NOD2 mutation has been studied in relation to infective events in adults and infants of very low birth weight.[163, 164] In relation to the three NOD2 mutations; Brenmoehl *et al* observed increased sepsis related mortality rate from 31% in the wild type NOD2 patients, to 36% in the two missense mutations, to 57% in the frameshift Leu1007fsinsC patients.[164] In very low birth weight infants, sepsis rate was higher in the frame-shift NOD2 mutation, with sepsis rate of 33% vs 13.2% non-carriers, with a trend to repeated episodes and blood culture proven sepsis in this group[163] (table 1.4.). A recent study conducted in the ICU admitted patients

(774 pts) showed the presence of at least one variant of NOD2 have reduced the phagocytosis function by monocytes rendering patients at higher risk of bacteraemia than the patient with the wild type genotype.[175]

1.7.5.5. Malignancies

Innate immunity and altered host immune response to microbial agents and molecular patterns was implicated as a cause of increase risk of developing NHL[162] and *H. pylori* induced mucosa-associated lymphoid tissue (MALT) lymphoma in NOD2 mutant patients.[159] There are reports suggesting an association between NOD2 frameshift mutation and non-Crohn's disease colorectal cancer risk at >50 years of age,[156] and another study associated breast cancer with first degree relatives with lung cancer to NOD2 risk alleles.[160] Both studies were based on Polish population, and need further validation (table 1.4.).

1.7.5.6. Autoimmune diseases and Inflammatory Diseases

A number of autoimmune diseases have shown linkage to the pericentrometric region of chromosome 16; including NOD2, disease such as rheumatoid arthritis and systemic lupus erythematous (SLE). As a result various investigators have tested specific diseases for the common CD NOD2 mutations.[147] No reports so far exist suggesting an association between NOD2 and rheumatoid arthritis,[172] SLE,[168] Wegener's granulomatosis,[176] Behcet's disease,[169] or MS.[170] NOD2 mutations have been associated with susceptibility to psoriatic arthritis[165] but not to ankylosing spondylitis.[171] Sarcoidosis had showed no association with NOD2 mutation in its asymptomatic naturally disappearing form, but this association was positive with the early progressive form of the disease.[154]

There seems to be an association with increased IgE levels, allergic rhinitis and atopic dermatitis.[166] For certain polymorphisms there is an association with protection against development of asthma of allergic rhinoconjunctivitis.[177] With

regards to infectious diseases, no association has been identified between NOD2 gene mutations and pulmonary tuberculosis[173] or periodontitis[174] (table 1.4).

1.8.　　This study

Genetic variants resulting in subtle changes in healthy individuals might be of greater significance in immunocompromised patients. We have chosen to study two genes (chitotriosidase and NOD2) involved in innate immunity and their influence on the risk of serious infections.

Innate immunity is a rapid and general response to injury. It relies on the detection of microbial motifs by presenting cells, such as macrophages, neutrophils and NK cells.

Phagocytosis, triggers both degradation of pathogens and presentation of antigens, stimulation of membrane bound receptors, TLR, by microbial components triggers expression of several genes involved in immune responses and activation of innate immune system.[94, 137]

Toll like receptors (TLR's)) are a family of pattern recognition receptors that sense motifs found in microbial pathogens called pathogen associated molecular patterns (PAMPS) and initiate immune defense against those pathogens. The majority of TLR's are expressed on the surface of myelomonocytic cells. The recognition of PAMPS by toll like receptors leads to the activation of the NF-κB signalling pathway.[94, 137]

Phagocyte specific chitotriosidase plays a role in innate immunity by degradation of chitin-containing pathogens (fungi, nematodes and insects).[106] This enzyme is located in specific granules of human PMNs and macrophages and rises in response to various infectious stimuli.[98, 99] Chitotriosidase showed increased activity in neonates not only with fungal but also with bacterial infections reflecting

phagocyte activation.[6] Patients with mutant allele and the resulting decrease enzyme activity may have increase susceptibility to such infections.[102]

The microbial motifs (PAMPs), LPS from the gram negative bacterial wall[138] and the PGN found in bacterial wall of both gram negative and gram positive,[139] are also recognised by the cytoplasmic proteins (NOD2) as a part of their innate immunity role.

A recent study showed that the NOD2 mutation reduces the phagocytic activity of monocytes and has a possible role in increasing the risk of bacteraemia. This risk was synergistic with the presence of mutant TLR.[175]

In another study, the release of chitotriosidase from specific granules in neutrophils occurred following PGN stimulation, and this was contributed to TLR and MDP-dependent innate immune response, while NOD2 activation induced chitinase expression in macrophages.[97]

As mentioned above chitotriosidase and NOD2 could play a role in innate immunity via their effects on the phagocyte specific regulation, with the contribution of TLR.

Patients with the diagnosis of haematological malignancies become neutropenic and immunocompromised due to chemotherapy. In this period they become highly susceptible to infections with different organisms which may have a serious impact in their prognosis.

In this study we looked into the innate immune genes, chitotriosidase and NOD2 in order to try and find out the weight of defect in the immune system that can be produced by their mutation. We tried to find a relation between susceptibility to infection, prevalence of organisms, and infection outcome. We first evaluated this relationship for each gene separately before determining if the presence of both mutations in an individual has a negative synergistic effect on the immune system.

Neutrophils are phagocytic cells containing potent antimicrobial peptides and proteolytic enzymes. They play an important role in acute inflammatory response, resolution of inflammation and assisting in the killing and digestion of

microorganisms. They are important for the clearance of infected foci before the appearance of macrophages, the chronic response cells.[178, 179] In this study we focused on patients who were likely to be rendered neutropenic by chemotherapy and / or their disease. By looking at the natural history of infection during the neutropenic phase it might be possible to uncover a relationship of to mutations in NOD2 and chitotriosidase when the patient is at their most vulnerable to effects on the innate immune system.

Chapter 2

Materials:

See appendix-5

General Methods

2.1.　　　　Study approval:

The study (genetic determinants of sepsis in haematological malignancy) was ethically approved by the central office for research ethics committees (REC Ref: 06/Q0501/56-Date 3/4/06. R&D ID:7414-Date 28/6/08).

2.1.1.　　　　Study population:

Patients were recruited from the Royal Free haematology in-patient wards initially. Later recruitment involved the day care unit and out-patient department at the Royal Free Hospital (RFH).

2.1.2.　　　　Inclusion criteria:

Specific criteria for recruitment of patients were applied and were as follows:

1. Age: 18 years or older.

2. Diagnosis of acute leukaemia, lymphoma, myelodysplasia, multiple myeloma or aplastic anaemia.

3. Able to give informed consent.

2.1.3. Exclusion criteria:

The following exclusion criteria were applied while recruiting patients in this study.

1. Age below 18 years.

2. Non-haematological malignancy.

3. Globally immunocompromised patients, including those with HIV/AIDS and agammaglobulinaemia.

4. Patients receiving immunosuppressive treatment except as explicitly part of their chemotherapy for their haematological malignancy. Patients included in this criterion are patients with organ transplant and allogenic bone marrow transplant.

5. Other severe co-morbidity, including congestive cardiac failure grade III (New York heart association classification), severe obstructive airways disease, Child B or C liver failure, short gut syndrome, active inflammatory bowel disease or liver disease.

6. Patients who were considered by the haematology team to be in a terminal phase of their condition.

7. Unable to give informed consent.

2.2. Patient recruitment

2.2.1. Case finding:

The clinical research fellow (LS; myself) attended the weekly hematology ward round to prospectively ascertain new cases, and follow up established cases including admissions to the hematology inpatient wards (Compston and Calthorpe wards).

Later in the course of the study patients were recruited from the hematology day care unit (OACS) and the outpatient department to enhance recruitment. In the case of those patients who had already commenced chemotherapy, data was collected from the date of their initial presentation and the diagnosis of malignancy.

2.2.2.   Patient approach and consent:

The total number of patients approached by the clinical research fellow (LS) was 310. 257 patients were initially deemed eligible and consented to the study. After scrutiny of available data and their history a further 53 patients were excluded. Unavailable data was the cause of exclusion in 20 patients, either due to long history or patients referred from other hospitals. Other reasons are listed in table 2.1.

Table   2.1.   Final disposition of patients enrolled in the study.

Causes of exclusion	Number of patients
Eligible for the study	204
Allogenic bone marrow transplant	22
Unavailable data	20
Not on chemotherapy (under observation only)	8
Immunosuppression (HIV, or post-transplant)	3 (1,2)

All patients deemed to fulfill the inclusion criteria were approached by the clinical research fellow, introduced to the study, and provided with written explanatory materials of a patient information sheet, consent form and an invitation letter (appendix-1). The patient was given at least 24 hours (depending on the patient's circumstances and general condition) prior to consent. The patient and relatives were allowed the opportunity to ask for more questions before handing back the consent.

2.2.3.   Follow up and data collection:

All patients who consented to participate in the study had a single 10ml anti-coagulated blood sample drawn during routine clinical venepuncture initially. In some cases the anticoagulated sample drawn for full blood count testing was utilized to avoid further venepuncture. Later and due to difficulty of obtaining sufficient DNA (immunosupressed and/or neutropenic), arrangements were made by the clinical research fellow (LS) to collect samples obtained from patients for complet blood count from the haematology laboratory. For most patients multiple samples (3-5) were needed necissating multiple visits to the laboratory. The processing of the sample is described separately.

Follow up of the patients in hospital was recorded by the clinical research fellow (LS) in a clinical record collection data sheet (appendix-2), with the patient demographics (hospital number, sex and date of birth), haematological diagnosis, chemotherapy (myelosuppressive and myelostimulation), date of admission and discharge. The following were documented daily- white cell and absolute neutrophil count, highest and lowest daily body temperature, any diagnosis of sepsis; including details of the source and results of microbiological culture and imaging investigations, and antibacterial and antifungal therapy. There was no necessity to interview or examine the patient.

The outcome of inpatient hospital stay (i.e. survival, resolution of septic episodes) was recorded at the end of the stay. Follow up of all the patients was performed using paper and electronic records until the study was concluded (appendix-2). For patients recruited prospectively the follow up until death or end of the study ranged from 5-22 months. For patients who had already commenced chemotherapy at study entry follow up extended up to 4 years.

2.3.   Laboratory analysis of blood samples:

A 10ml EDTA anticoagulated blood sample was processed, RBCs were lysed, the DNA was extracted using Puregene DNA Purification kit, and stored frozen at (-20)°C all done by the clinical research fellow (LS). The DNA was used to determine the genotype of the NOD2 and chitotriosidase genes using PCR-based techniques.

2.4.   Data Analysis:

At the end of each hospital visit patient data sheets (appendix-2) were collected by the clinical research fellow (LS) for data extraction and analysis. The following variables were collected: daily absolute neutrophil count with note of any neutropenia (neutrophil count below 0.5×10^9/L) and the duration of neutropenia in days. Febrile episodes based on RFH protocols (fever of 38°C in two occasions within 24 hours, fever of 38.5°C in one occasion). Presumed or determined source of sepsis; intravenous catheters (peripheral or central), respiratory or gastrointestinal. Any documented bacteraemia, fungaemia or viraemia within or outside of a neutropenic episode. Relevant imaging findings (including CxR, CT, USG abdomen). Details of antimicrobial / antifungal therapy and response. Survival to discharge, or death in hospital. NOD2 and chitotriosidase genotype.

In cases where the discharge occurred before the end of antimicrobial therapy, the outcomes were determined by following the patient's records. There was no necessity for patients to attend the hospital solely for this study.

Analysis was performed using Microsoft office excel 2007. Univariate statistical analysis was used to determine the potential association of genetic mutations with increased risk of fever, bacteraemia, fungaemia, and adverse outcome following sepsis. Data was also analyzed separately for those patients who were neutropenic and those who were not. Frequency distribution analyses were done by Chi squared and Fisher exact test using web calculator. P value of ≤0.05

considered significant. (http://www.physics.csbsju.edu/stats/fisher.form.html) (http://www.physics.csbsju.edu/stats/contingency_NROW_NCOLUMN_form.html)

2.5. Confidentiality:

Complete patient confidentiality was maintained at all times. No personal data was stored in connection to samples. Only the clinical research fellow (LS) and immediate supervisor had access to raw data. All samples were stored in secure freezer in a secure laboratory and all remaining material was destroyed at the end of the study.

2.6. Red cell lysis solution:

Whole blood was placed in a sterile falcon tube, labeled with a unique laboratory identification number. The sample was diluted by adding PBS 1:1 and centrifuged for 10 minutes at 1400 rpm. The supernatant was removed; the pellet was dislodged and the tube was topped up with RBC lysis solution (Gentra Puregene; Wales;UK). Sample was then incubated on ice for 20-30 min and centrifuged to produce a white pellet. Latter the supernatant was discarded and the pellet dislodged. Steps were followed by the clinical research fellow (LS) as per the manufactures' guidelines.

2.7. Extraction of DNA:

After breaking up the pellet, the cell lysis solution (Gentra Puregene; Wales;UK) was added to the cells. The amount of cell lysis solution was noted and left overnight. Protein precipitation solution (Gentra Puregene; Wales;UK) was added at one third of the volume of cell lysis and incubated in ice for 20-30 minutes. 1ml aliquots were dispensed into 1.5ml microcentrifuge and centrifuged at 13000 rpm

for 10 min. A pellet was formed with a clear supernatant. The supernatant was poured into a labeled 15 ml tube and isopropanol (equal in amount to cell lysis solution) was added. The tube was inverted until the DNA started to precipitate. Excess Isopropanol was removed and 1ml isopropanol with the DNA was poured into a new labeled 1.5ml microcentrifuge tube and centrifuged at 13000rpm for 1 minute. DNA appeared as a white pellet at the bottom after being centrifuged.

Excess isopropanol was discarded and 1 ml of 70% ethanol was added to the DNA pellet and centrifuged at 13000rpm for 1 min and then poured out. The DNA pellet was left air dried at room temperature, by covering the opening of the tube with some pierced film leaving it overnight. Once dry, at least 50µl of double distille water (Gentra Puregene; Wales;UK) was added to the DNA pellet and allowed to resuspend.

The DNA concentration was checked by the clinical research fellow (LS) using the Nanodrop according to the manufacturer's protocol. If the concentration was greater than 200ng/µl, then it was brought down by further addition of DNA hydration solution. The DNA concentration was written on worksheet and at the side of the tube and stored at -20°C. Steps were followed as per the manufactures' guidelines.

2.8. Chitotriosidase genotyping:

2.8.1. <u>Stock regents:</u>

Oligonucleotide Primers (Sigma Genoys; Texas;USA) arrived lyophilized and were reconstituted with ddH$_2$O as specified on the certificate of analysis to a 100µM concentration. Primers were then diluted 1:10 in ddH$_2$O to give working concentration of each primer, and stored at (-20)ºC.

2.8.2.   Chitotriosidase Genotyping Process:

PCR was carried out in a total volume of 23µl per reaction, containing 2.5µl of 15 mM $MgCl_2$, 2.5µl of 10x Reaction Buffer, 0.5µl of 10 mM of each primer (chitotriosidase F and R), 0.5µl of 2mM dNTP, 0.25µl of (5 µ/µL) taq polymerase (Bioline ltd; London;UK), and 16.25µl ddH_2O to make up to the final volume.

2µl of 100-200 ng/µl genomic DNA was added to the appropriate tubes and 2µl of sterile water to the blank tube and centrifuged. The PCR conditions for chitotriosidase were as follow: Denature 96ºC for 5 minutes runs for 1 cycle. This was followed by 35 cycles of; denaturing at 96ºC for 45 seconds, annealing at 61ºC for 45 seconds followed by extension at 72ºC for 45 seconds. The final extension was carried away at 72ºC for 10 minute for 1 cycle followed by 4ºC in finitely.

20µl of each PCR product was mixed with 4µl of bromophenol blue loading buffer to promote visualization on the gel.Gel electrophoresis was performed on 1.5% agrose gel (0.75gram agrose powder (Bioline ltd; London;UK), in 50ml of 0.5 TBE, 5 µL ethidium promide) in 0.5xTBE buffer at 150V for one hour. 1.5µl of the DNA ladder (Bioline ltd; London;UK) was loaded into the last well. The gel was photographed on the gel imaging system (Gel Doc 2000 (BioRad; California, USA)) and results were interpreted (figure 2.1.).

1   2   3   4   5   6   7   8   9   10   11   12
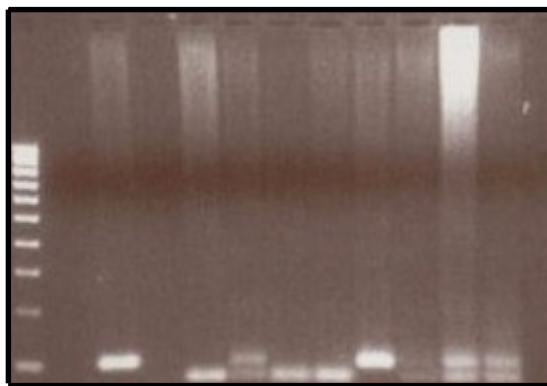
Figure 2.1. Chitotriosidase genotyping: Gel visualization of PCR fragments size; 1; The DNA ladder (IV), 2 The blank (ddH_2O) control, 3 The positive control (GD; homozygote for the mutation), 5,7,8 are wild type chitotriosidase. 6,11,12 are heterozygote for chitotriosidase. 9 is homozygote.

PCR fragments sizes are: wild type chitotriosidase 75 basepair (bp); heterozygous for chitotriosidase mutation at 75bp+99bp and homozygous for chitotriosidase mutation at 99bp. The clinical research fellow (LS) carried out the methods as per the manufactures' guidelines.

2.9. NOD2 genescanning

2.9.1. <u>Stock regents:</u>

Oligonucleotide Primers (vh bio; Edinburgh; UK) arrived lyophilized and were reconstituted with sterile water as specified on the certificate of analysis to a 100µM concentration. Primers were then diluted 1:10 in ddH$_2$O to give working concentration of each primer, and stored at (-20)ºC.

2.9.2. NOD2 Genescanning using ABI 3130

PCR was carried out in a total volume of 19µl, and two reactions were prepared in 1.5 microcentrifuge tubes. The 1st reaction containing the common exon and the wild type primer and the 2nd reaction contains the common exon and the SNP primer. Master mix reagents are prepared as follow; ddH$_2$O 11.9µl, 10x buffer 2µl, 15mM MgCl$_2$ 2µl, 2mM dNTPs 1µl, either primer WT or SNP (0.4 µM) 1µl, Common primer (0.4 µM) 1µl, last taq polymerase 0.1µl.

Two sets of 250µl thin walled PCR tube were labeled for each primer mix (WT/SNP). Each containing the number of samples to be tested, and two controls: positive control either mutant for the SNP set or wild type, and a blank.

In each tube 2µl of 100-200 ng/µl genomic DNA was added to the appropriate tubes and 2µl of ddH$_2$O to the blank tube. All tubes were briefly centrifuged and placed into the PCR machine / thermal cycler PCR conditions applied to all were the same except for the annealing temperature. Denature 94ºC for 5 minutes for 1 cycle, then 25 cycles of denature at 94ºC for 30 seconds, annealing at xºC (table

Table 2.2.   Annealing temperature for each exon in NOD2 genotypes.

Annealing temp	Wild type	SNP
Exon4 (SNP8)	69°C	65°C
Exon8 (SNP12)	65°C	65°C
Exon11 (SNP13)	70°C	65°C
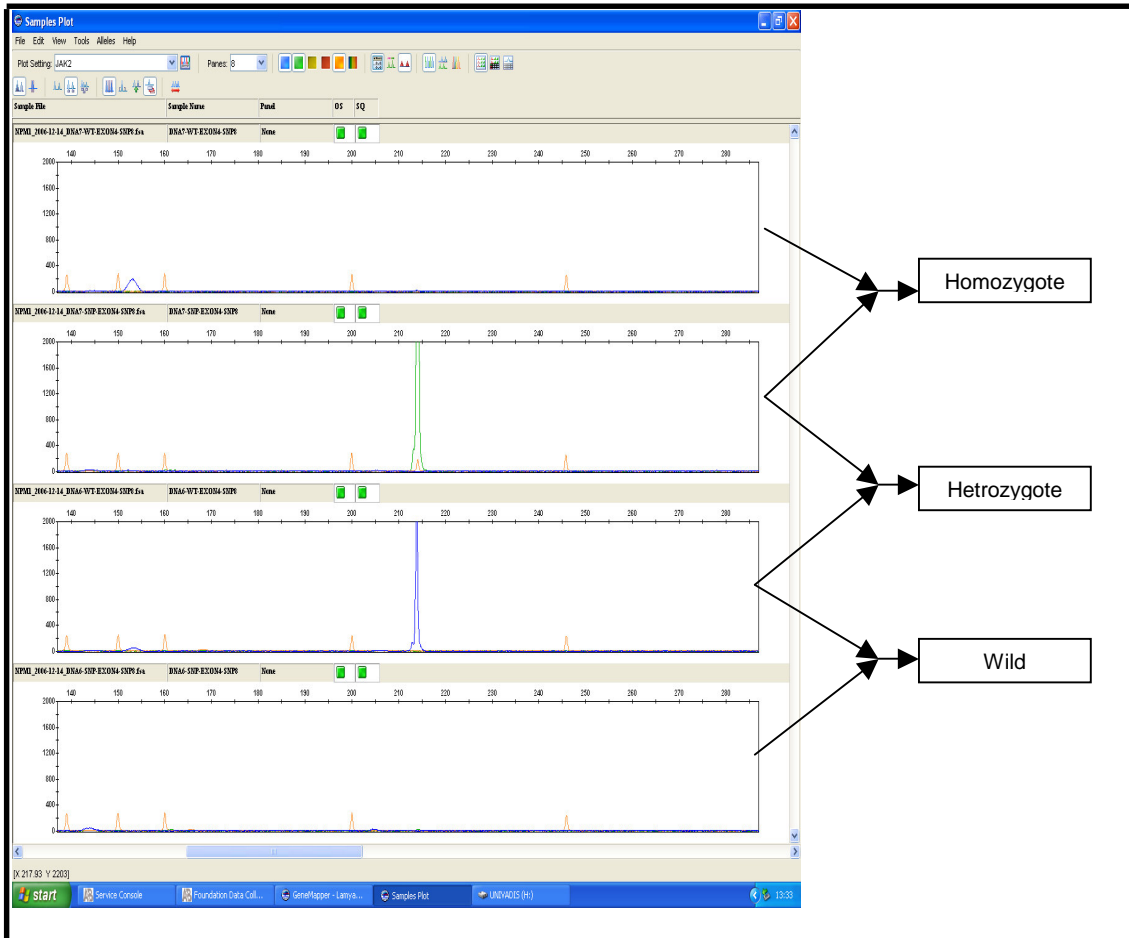
Figure 2.2.   Genescan trace figure: shows typical trace of genescan assay.
The x axis shows size of the fragment. The y axis shows intensity.
The small orange peaks are the size standard, represent fragments of different sizes.
The colour of the peak is dependent upon which base is present at the loci of interest as the two forward primers (Wild type or SNP) are fluorescently labelled.
The first 2 lines show a homozygote mutation with a green peak.
The last 2 lines are wild type NOD2 genotype with the blue peak.
The middle 2 lines, where the two primers peaks (green and blue) appear it indicate heterozygote mutation.
The missenses and frameshift mutations are both detected by genescanning in the same manner.

2.2.) for 30 seconds followed by extension at 72⁰C for 45 seconds. The final extension was 72⁰C for 5 minute to run 1 cycle, followed by 4⁰ C in finitely.

For genescanning, a mixture was prepared of 10µl HiDi formamide (AB applied Biosystems; California;USA) and 0.75µl of size standard (AB applied Biosystems; California;USA) vortex mixed before and frequently while pipetting. 10.75µl of the mixture was placed into the wells of a PCR microplate (96-well plate suitable for ABI 3130) (Axygen Scientific; California;USA) suitable for use in a machine (ABI 3130) capable of fragment analysis via capillary electrophoresis), followed by 1µl of PCR product.

The plate was denatured at 94⁰C for 5 minutes and placed in the plate base (AB applied Biosystems; California;USA) with plate septa (AB applied Biosystems; California;USA) and a plate retainer (AB applied Biosystems; California;USA), within the appropriate instrument for fragment analysis. The polymer-3130 POP7 (AB applied Biosystems; California;USA) was used containing 3.5ml or 7ml (figure 2.2). The capillary electrophoresis analyzer was operated by the clinical research fellow (LS) as instructed in the manufacturer's handbook.

2.10.   Sequencing of PCR products[180] using ABI 3130

The DNA template was first labelled by incorporation of four fluorescent dyes (one for each dNTP) using a PCR primer extension technique. Labelled DNA was then electrophoresed through a 34cm capillary tube containing a premade polymer.

Two PCR reactions were prepared in 1.5ml centrifuge tubes each. The 1st reaction contains the reverse primer and the 2nd reaction the forward primer. Master mix regents were prepared as follows; sterile water 5.5µl, 5x sequencing buffer 2µl (AB applied Biosystems; California;USA), Big Dye (version 1.1, Applied Biosystems) 0.5µl, and 1µl of either primer (forward or reverse). In a 96-well plate suitable for ABI 3130 master mix of 9µl was added to each well. The DNA amplicon sample was diluted in double distilled water 1:30 and then 1µl was added to the master

mix. The plate was sealed firmly with adhesive film (AB applied Biosystems; California;USA) and left to spin down in centrifuge.

A full plate cover (AB applied Biosystems; California;USA) was placed on top of the plate, and then placed in a hot-lidded PCR machine-Tetrad & Dyad blocks (MJ Research;Aberdeen;UK) PCR sequencing amplification was carried out as follows: 95°C for 4minutes to run 1 cycle, 24 cycles of 95°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes.
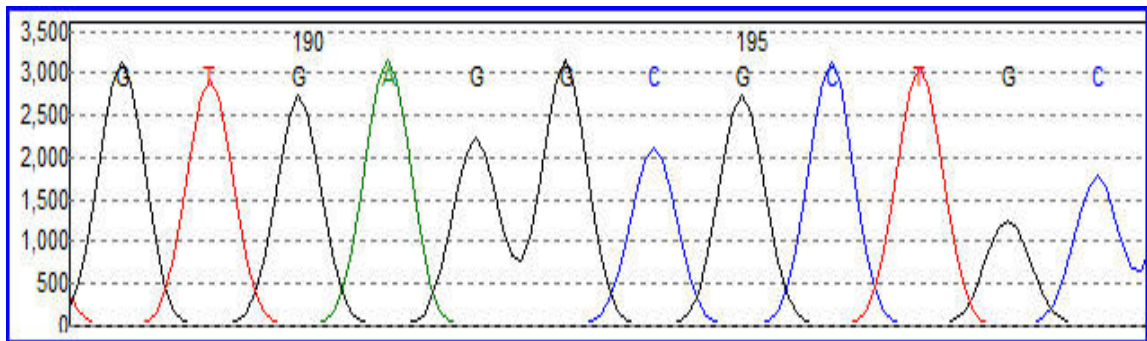
Figure 2.3. DNA sequencing: The term DNA sequencing refers to methods for determining the order of the nucleotide bases, adenine, guanine, cytosine, and thymine, in a molecule of DNA. DNA sequencing technology using dye-based (DNA is labelled by incorporation of four fluorescent dyes) sequencing methods with automated analysis.

To ensure desalting and removal of the remaining labeled dNTPs and unincorporated dye terminators 2μl of 3Mmol Na Acetate, 2μl of 125Mmol EDTA and 50μl of 100% Ethanol was added to each well and left at room temperature for 15 minutes before spinning the plate at 3400 rpm for 30 minutes at 10°C. The plate was immediately inverted on a towel in the plate holder of the centrifuge and pulse spun inverted till the machine reached 1000 rpm. DNA was then washed with 70μl of fresh 70% Ethanol (stored at -20°C) and spun at 3400 rpm for 15 minutes. The plate was immediately inverted on a towel in the plate holder of the centrifuge and pulse spun inverted till the machine reached 1000 rpm. Pellets were allowed to dry on the top bench for 15-20 minutes, while the manager/sample sheet was prepared. The Ultra run programme was used if the product size was less than 500bp, or rapid run programme if the size was greater than 500bp, .Before running

the plate, the samples were resuspended in 10µl HiDi formamide by pipetting it up and down about 5-6 times with the sample in the first four wells, of the first run. Mixing was not required for the rest of the wells since HiDi formamide denatured samples in 20-25 minutes. Plates were stored at -20°C until ready to run and only re-suspended in 10µl of HiDi formamide just before running the samples. Before running the samples, the plate was assembled along with the black plate base, a plate septa and a plate retainer. For ABI 3130 sequencing machine the polymer-3130 POP7 was used containing 3.5ml or 7ml (figure 2.3.).

2.11.   Pyrosequencing

Primers for the pyrosequencing assays were designed using Pyrosequencing Assay Design Software 1.0.6. (Biotage Ltd). Oligonucleotide Primers (vh bio; Edinburgh;UK) arrived lyophilized and were reconstituted with sterile water as specified on the certificate of analysis to a 100mM concentration. Primers were then diluted 1:10 in ddH$_2$O to give working concentration (10mM) of each primer, and stored at (-20)°C. Each primer set consisted of a Forward (F1), a Reverse (R1) and a Sequencing Primer (S1).

The PCR reaction was prepared in a total volume of 20µl, containing 2µl of 15mM MgCL$_2$ and 10xbuffer. 1µl of 2mM dNTP, 1µl of each 10mM primer (F1/R1), 0.1µl taq polymerase, 11.9µl of ddH$_2$O and 1µl of 100-200ng/µl genomic DNA.

The solution was incubated in the G-Storm thermal cycler (Biotage;Virginia;USA)at 94°C for 3 minutes, followed by 32 cycles of denaturing 94°C for 40 seconds, annealing 60°C for 40 seconds and elongation 72°C for one minute. Followed by 10 minutes elongation at 72°C and stored in finitely at 10°C.

All PCR products were analysed by standard gel electrophoresis on a 1.5% agrose gel with Sybersafe^{TM} DNA gel stain (invitrogen; Oregon;USA) prior to pyrosequencing figure 2.4.).

1 (2-8)   9 (10-16)
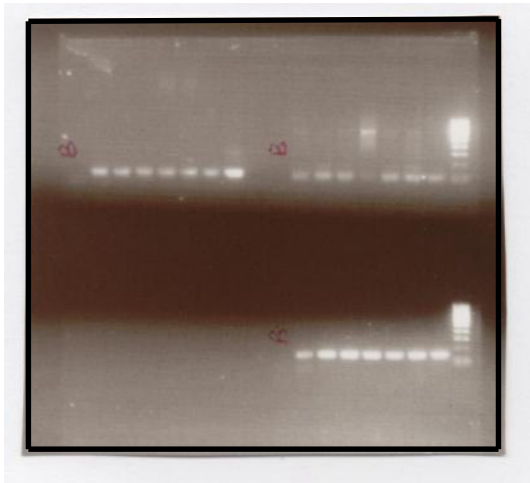
Figure 2.4. NOD2 PCR product visualization: In the 1.5% agrose gel at 100bp for exon 4, and 8 while 150bp for exon11, hyperladder IV was used. B for blank (ddH$_2$O). 1 and 9 are wells containing blanks. Wells (2-8) are exon 4 products, and (10-16) are exon 8 products. The lower row is products of exon 11 along with the blank.
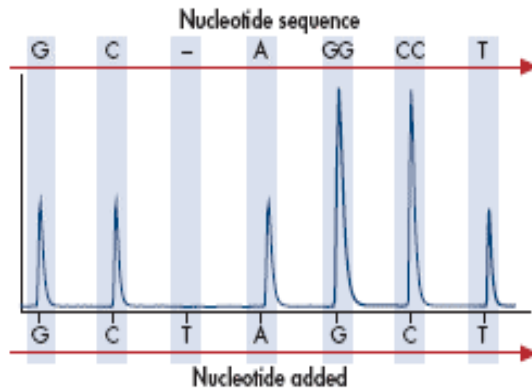
Figure 2.5. Pyrosequencing trace.The dATP (dNTP component) is used as a substitute for the natural deoxyadenosine triphosphate (dATP) since it is efficiently used by the DNA polymerase. As the process continues, the complementary DNA strand is built up and the nucleotide sequence is determined from the signal peaks in the Pyrogram trace (adapted from the web)

Pyrosequencing is an alternative to geneScan, and allows nucleotides at the loci of interest to be quantified. The assay involves a Binding Reaction containing 15-20 µl PCR product, 38 µl of Binding Buffer (Biotage;Virginia;USA), 2 µl of Streptavidin Sepharose beads (GE healthcare; Sweden) and 20 µl of ddH$_2$0. The plate is shaken for 15 min in a plate shaker (WebScientific; Cheshire;UK) at 1300 rpm. Following this, the purification step involves washing with 0.2M NaOH, 70% Ethanol and 10X Washing Buffer (Biotage) according to the manufactures'

guidelines. The final step before analysis is the Annealing reaction; 24.2 µl Annealing Buffer (Biotage) and 0.8 µl of sequence primer (S1) are aliquoted in to PyroMark™Q24 Plates (Biotage) and the bound Streptavidin Sepharose-PCR product is placed in the wells. The plate is heated at 80°C for 3 minutes and then placed in the PyroMark Q24 (Biotage) for SNP analysis as per the manufactures' guidelines by the clinical research fellow (figure 2.5.).

Chapter 3

Chitotriosidase as genetic determinant of sepsis in haematological malignancy

3.1.   Introduction

Infection remains a significant cause of morbidity and mortality in patients with haematological malignancy. This may be a result of either the effect of the disease process itself on defensive mechanisms or as a sequel of chemotherapy. Other risk factors include immune suppression, mucosal damage, and indwelling catheters. Bacterial pathogens and fungal infections are associated with increased risk of mortality.[96]

It is possible that genetic variation may result in heterogeneity in the proinflammatory cytokine profile disturbing the balance of circulating cytokines and influencing the inflammatory response thereby rendering an individual more susceptible to infections.[96]

Human Chitotriosidase is a fully active chitinase, expressed by activated macrophages. It was first discovered in the plasma of patients with Gaucher disease.[7]

Gaucher disease is the most prevalent lysosomal storage and results from an autosomal recessive defect, leading to deficient activity of lysosomal hydrolase, β-glucocerebrosidase. The accumulation of glucocerebroside occurs within the lysosomes of macrophages resulting in accumulation of Gaucher cells within the reticuloendothelial system especially liver, spleen and bone marrow. There are three types of GD, the commonest is type 1 where presentation is heterogeneous and slowly progressive. The symptoms developing towards adulthood include

haematological abnormalities, skeletal pathology and hepatosplenomegaly. Type 1 disease has historically been defined by the absence of central neurological involvement however recently an association with Parkinson's disease has been detected. Types 2 and 3 are rarer forms of the disease and manifest significant neurological features. The onset in the most severe type 2, is at infancy 3-6 months with death before the age of 2 years. Type 3 manifests in childhood and although less severe than type 2, treatment usually fails to improve the neurological symptoms. Haematological and skeletal manifestations may also occur.[116]

Human polymorphs and mature macrophages are the main source of chitotriosidase, being secreted from their lysosomes.[7] Chitin is a major structural component of the cell walls of fungal pathogens,[181] analogous to some plant chitinases, recombinant chitotriosidase has been found to inhibit hyphal growth of chitin-containing fungi such as *Candida* and *Aspergillus*, and improved survival in neutropenic mice models with such systemic fungal infections.[7, 106, 182] The role in degradation of chitin-containing pathogens as antifungal action and the chitotriosidase expression by phagocytes suggests a physiological role in defence against such infections.[106, 182]

Immune compromised individuals show an increased risk of life-threatening fungal infections. Induction of chitotriosidase expression in the mature macrophages and PMNs following GM-CSF treatment had shown improved anti-fungal response by inhibiting growth of *C. neoformans* and *C. albicans* due to the chitinolytic activity towards the cell wall chitin.[7] Chitotriosidase has been found to be elevated in plasma of neonates with systemic candidiasis,[6] and associated with bacterial infection in bone marrow (BM) transplant and in endemic areas for filarial parasite.[115] It is up-regulated both at the level of RNA and protein activity following stimulation with prolactin, IFN-γ and TNFα.[109] IL-12 injection in chimpanzees is also associated with enhanced chitotriosidase activity. This suggests a possible role of chitotriosidase during responses to infection.[114]

Chitotriosidase deficiency is inherited as autosomal recessive and is multiethnic occurrence.[106] About 30-40% Caucasian population bears the mutation caused by24 duplication in exon 10 in which 6% are homozygote.[106, 107] Another two polymorphisms (G354R and A442V) are found predominantly in African decent and are associated with reduced chitotriosidase activity.[108]

In the present study we examined the presence or absence of chitotriosidase mutation in patients with haematological malignancies, as patients with such diagnosis are prone to neutropenia and are rendered immunocompromised by treatment. Evaluation of chitotriosidase mutations in relationship to infection and sepsis was studied in this group of patients in relation to absolute neutrophil count (neutropenic or non-neutropenic). The infectious pathogen and outcome of infection were analysed according to mutant status immune defences and the susceptibility of developing bacterial and fungal infections.

3.2.   Hypothesis:

Our hypothesis is that patients carrying mutations in chitotriosidase, a mediating innate immunity gene, are at increased risk of infection and sepsis during periods when they are neutropenic.

3.3.   Objectives:

1) To determine the effect of chitotriosidase gene status on the incidence and outcome of sepsis in patients with haematological malignancy, including those with leukaemia, lymphoma, and myeloma.

2) To compare the effect of chitotriosidase gene status on the incidence and outcome of sepsis in patients with haematological malignancy who are neutropenic and those who are not.

3) Prevalence of organisms.

4) Correlate neutrophil function and duration of neutropenia with the chitotriosidase genotypes.

3.4. Materials and methods:

General material and methods are described in Chapter 2.

Inclusion and exclusion criteria are also described in chapter 2.

Recruitment of patients with haematological malignancy was performed in the RFH.

The study was ethically approved by the central office for research ethics committees (REC Ref: 06/Q0501/56-Date 3/4/06. R&D ID:7414-Date 28/6/08). All patients gave informed consent. They were followed up during their in-patient stay. Clinical data were collected daily by the clinical fellow. Observations were recorded including ANC, temperature, related investigations (microbial and imaging) to determine the source and type of sepsis, and antimicrobial management.

Investigation and data of patients recruited from the out-patient department or after patients discharge were recorded electronically or from paper medical records by the clinical research fellow (LS).

DNA was extracted and genotyped for chitotriosidase, according to methods mentioned in chapter 2.

3.5. Statistics:

P value was calculated by the Chi square, and by Fisher exact if figures $\leq$ 10 if comparing two dimensions. P value of ≤ 0.05 considered significant.

(http://www.physics.csbsju.edu/stats/contingency_NROW_NCOLUMN_form.html)

(http://www.physics.csbsju.edu/stats/fisher.form.html)

P value differences were calculated in four proportions. The Wild type: Heterozygote: Homozygote proportion is mentioned in this chapter while the Wild type: Mutant, Wild type: Heterozygote, Wild type: Homozygote proportions are mentioned in appendix 4.

Mean, median and calculation along with graphs were prepared using Microsoft excel.

3.6.   Results:

3.6.1.   Incidence of chitotriosidase mutation:

Mutation: Out of the 204 patients, 122 (59.80%) were wild type for chitotriosidase and 82 (40.20%) were mutated. Of the 82 mutant genotype patients 71 (34.80%) were heterozygote and 11 (5.39%) were homozygote for the mutation (table 3.1.).

3.6.2.   Demographic characteristics:

A total of 204 patients were included in this part of the study. Males accounted for 59.31% and females for 40.69% (p=0.443). The age ranged between 18-81 years at the time of diagnosis (table 3.1). The age and gender distribution did not differ between wild type and mutated genotype patients.

3.6.3.   Range of Diagnoses

In this study population the diagnosis of lymphoma accounted for 52.45%, acute and chronic leukaemia (lymphoblastic and myeloid) for 30.39%, multiple myeloma and plasma cell malignancies for 15.69%, and other malignancies for 1.47%. There

were no significant differences in the diagnosis between the genotypes (table 3.2. & figure 3.1).

Table 3.1. Demographic characteristics in the study population. The number of patients and proportion by sex and age at diagnosis of haematological malignancy in relation to chitotriosidase gene status. (M: male, F: female)

Chitotriosidae gene status	Sex		Age at diagnosis							
	M	F	18-20	21-30	31-40	41-50	51-60	61-70	71-80	81-90
Total; 204 %	121 59.31	83 40.69	5 2.45	19 9.31	24 11.76	28 13.73	53 25.98	49 24.02	23 11.27	3 1.47
Wild type; 122 59.80 %	75 61.48	47 38.52	2 1.64	14 11.47	14 11.47	15 12.30	35 28.69	30 24.59	11 9.02	1 0.82
Mutant; 82 40.20 %	46 56.10	36 43.90	3 3.66	5 6.10	10 12.20	13 15.85	18 21.95	19 23.17	12 14.63	2 2.44

Table 3.2. Diagnosis of different haematological malignancies in our study population. The number of patients and frequencies (%) of the total in relation to the chitotriosidase gene status. The p value calculation by Chi square and Fisher exact tests comparing wild type: mutant.

Chitotriosidase status	Diagnosis				
	Lymphoma	Multiple myeloma	Acute Leukaemia	Chronic Leukaemia	Other
Total; 204 (%)	107 (52.45%)	32 (15.69%)	51 (25%)	11 (5.39%)	3 (1.47%)
Wild type; 122 (59.80%)	67 (54.92%)	17 (13.93%)	27 (22.13%)	8 (6.56%)	3 (2.46%)
Mutant; 82 (40.20%)	40 (48.78%)	15 (18.29%)	24 (29.27%)	3 (3.66%)	0
P	0.389	0.401	0.248	0.176	0.212

Table 3.3. Outcome of the patients included in the study in relation to chitotriosidase gene status. (CR: complete remission, PR: partial remission, Allo: allogenic transplant, On R: still on treatment at last follow up.)

Chitotriosidase gene status	Outcome						
	CR	PR	Allo	Death	On R	Relapse	Progressive
Total;204 %	60 29.4%	42 20.59%	33 16.18%	36 17.65%	17 8.33%	9 4.41%	7 3.43%
Wild type: 122 (59.80%)	34 27.8%	28 22.95%	19 15.57%	21 17.21%	11 9.02%	7 5.74%	2 1.64%
Mutant: 82 (40.20%)	26 31.7%	14 17.07%	14 17.07%	15 18.29%	6 7.32%	2 2.44%	5 6.10%
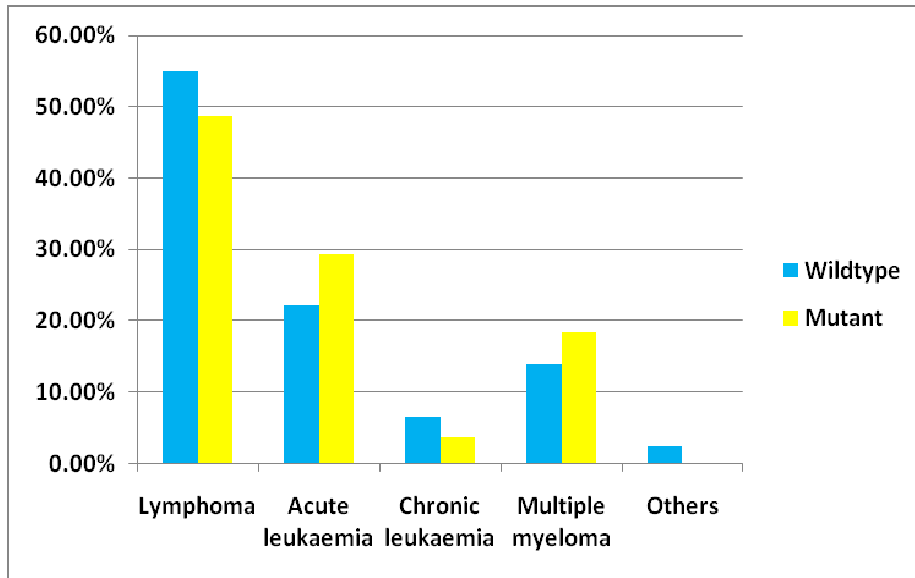
Figure 3.1.

Diagnosis of different haematological malignancies. Frequencies in relation to chitotriosidase genotype.
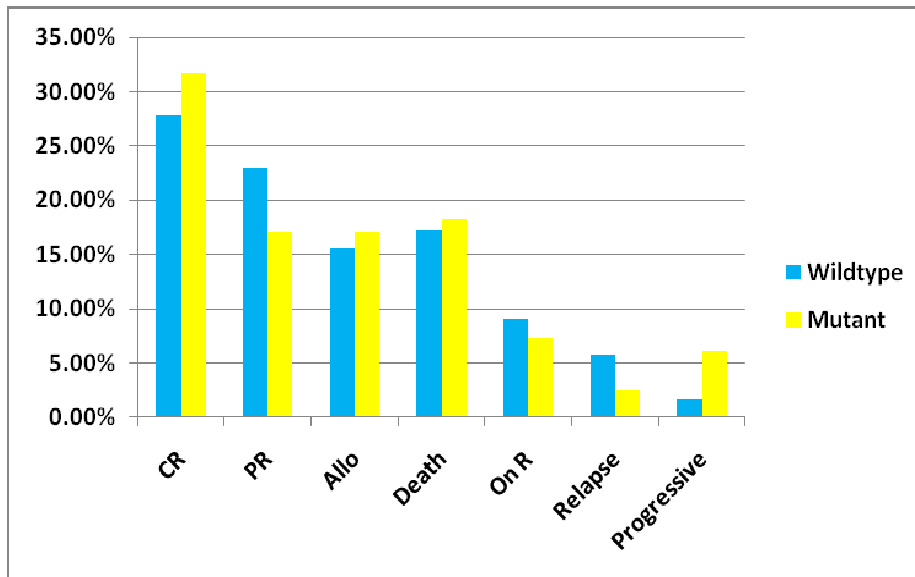
Figure 3.2.

Outcome of haematological malignancies in the study population in %, in relation to chitotriosidase genotype.

3.6.4. Outcome of the haematological malignancy in relation to chitotriosidase status

Out of the total 204 patients, 29.41% entered complete remission, 20.59% partial remission while 17.65% patients died due to the treatment or disease. 16.18% patients received allogenic bone marrow transplant, and 4.41% relapsed whilst the

study was still in process, 8.33% patients were still on treatment by the end of the study and data analysis. 3.43% of the patient responses were progressive or refractory to treatment. The different outcomes did not show any significant differences between chitotriosidase wild type and mutant genotypes. Complete remission (CR), allogenic transplant, deaths and progressive course, p=0.555, 0.776, 0.843 and 0.086 respectively (table 3.3. & figure 3.2.).

There may be a trend towards a progressive course of disease in patients with the chitotriosidase mutation (p=0.086).

3.6.5. Analysis of episodes of neutropenia (EoN):

Neutropenia was defined as an absolute neutrophil count (ANC) $\leq 0.5 \times 10^9$/L. Patients receiving chemotherapy became neutropenic during the course of therapy. Neutropenia was analysed according to its incidence and duration for each genotype. We also looked into the number of patients per genotype developing neutropenia and the frequency of neurtopenic episodes in these patients.

3.6.5.1. Number of patients with neutropenic episode per genotype:

The total number of patients developing episodes of neutropenia in the wild type group was 103 out of 122 (84.43%) whilst in the mutant genotype 65 out of the 82 patients (79.27%) became neutropenic during their treatment. 54 out of 71 (76.06%) of the heterozygote became neuropenic at some point, while all the homozygote 11 patients (100%) had one or more neutropenic episodes. There were no statistically significant differences between the chitotriosidase genotypes, (table 3.4.).

3.6.5.2. Number of neutropenic episodes per genotype:

There were 453 episodes of neutropenia in total, 282 episodes out of the 453 occurred in the wild type chitotriosidase patients, while 171 out of the 453

occurred in the mutant genotype patients. There was no statistical difference in the proportion of each genotype exhibiting episodes of neutropenia (table 3.5.).

The number of neutropenic episodes per patient was slightly higher in the mutant, homozygote but was not significant by statistical tests (table 3.5.).

Table 3.4.   Total number of patients (Pts) and total patients with episodes of neutropenia (EoN) in each chitotriosidase genotype. % of neutropenic patients within each genotype. The p was calculated by Chi square.

Total patients =204	Wild type =122	Mutant =82	Heterozygote =71	Homozygote =11
Total patients (%)	122 (59.80%)	82 (40.20%)	71 (34.80%)	11 (5.39%)
Pts with EoN in each genotype (p= 0.098)	103 (84.43%)	65 (79.27%)	54 (76.06%)	11 (100%)

Table 3.5.   Episodes of neutropenia (EoN) analysed by different chitotriosidase genotypes. The p value was calculated by t-test.

Total episodes of neutropenia = 453	Wild type	Heterozygote	Homozygote
Total pts / genotype	122	71	11
Total neutropenic episodes P (wild type: mutant)=0.21	282	135	36
Total neutropenic days P (wild type: mutant)=0.18	2845	1332	266
Range Episodes of neutropenia / patient Neutropenic days per patient	0-11 0-109	0-7 0-50	1-13 1-33
Median Episodes of neutropenia/patient Neutropenic days/patient Day/episode	2 13 6.5	2 10 5	2 19 9.5

3.6.5.3.   Duration of neutropenic episode:

The duration of neutropenic episodes varied, between patients. We calculated the total number of days of neutropenia for each genotype, to try to evaluate if the chitotriosidase mutation affected the time to neutrophil regeneration. The total

number of neutropenic days for each genotype were 2845, 1332 and 266 in the wild type, heterozygote and homozygote patients respectively (table 3.5.).

The number of neutropenic days per episode was slightly higher in the homozygote mutant genotype (median: 6.5, 5, and 9.5 days per episode for the wild type, heterozygote and homozygote respectively). The total duration of neutropenia per patient was longer in the mutated homozygote patients (mean 24.18 days, compared to median 19 days). Again, although suggestive, there were no statistically significant differences (table 3.5.).

Overall we found no statistically significant differences between the different chitotriosidase genotypes and the occurrence of neutropenic episodes.

3.6.6.   Analysis of febrile events:

A febrile episode was diagnosed, if fever of 38.5°C or greater occurred on one occasion or fever of 38°C occurred on two occasions (with or without neutropenia).

To determine if the occurrence of fever was affected by the chitotriosidase mutation. We analysed the incidence of febrile events per genotype then the number of patients in each genotype who development such febrile events in relation to presence and/or absence of neutropenia.

3.6.6.1   Febrile neutropenic events (FNE):

The total number of febrile events throughout all genotypes was 316; 202 febrile events occurred during neutropenia while 114 occurred in the absence of neutropenia. (table 3.6.).

There were no statistical differences in the proportion of patients developing febrile events, either neutropenic or neutropenic, or both for all genotypes (table 3.6.).

The number of febrile neutropenic events (event/neutropenic episode) per patient, per episode of neutropenia and per days of neutropenia was almost the same in all genotypes, p=>0.05 (table 3.7.).

Table 3.6. Total febrile events in each genotype. Proportions of patients (Pts) out of each chitotriosidase genotype with febrile events whilst neutropenic (FNE), non-neutropenic (FNnE), either (FNE or FNnE) or both (FNE & FNnE). The p value calculated by the Fisher exact test compares the wild type: heterozygote: homozygote proportions.

Total	Wild type	Mutant	Heterozygote	Homozygote
Total pt 204 (%)	122 (59.80%)	82 (40.20%)	71 (34.80%)	11 (5.39%)
Total Febrile events = 316 (%;events genotype/total event)	199 (62.98%)	117 (37.02%)	101 (31.96%)	16 (5.06%)
Total FNE = 202 (%;events genotype/total event)	126 (62.38%)	76 (37.62%)	63 (31.19%)	13 (6.43%)
Total FNnE = 114 (%;events genotype/total event)	73 (64.04%)	41 (35.96%)	38 (33.33%)	3 (2.63%)
Pts with FNE (% pts of FNE/ genotype). P = 0.466	73 (59.84%)	42 (51.22%)	36 (50.70%)	6 (54.55%)
Pts with FNnE (% pts of FNnE/ genotype). P = 0.834	47 (38.52%)	29 (35.37%)	26 (36.62%)	3 (27.27%)
Pts with either febrile events P = 0.559	56 (45.90%)	37 (45.12%)	30 (45.25%)	7 (63.64%)
Pts with both febrile events. P = 0.424	33 (27.05%)	17 (20.73%)	16 (22.54%)	1 (9.09%)

3.6.6.2. Febrile non-neutropenic events (FNnE):

114 events of non-neutropenic fever occurred. The number of patients with febrile events not associated with neutropenia were 47 out of the 122 in the wild type (38.52%) and 29 out of the 82 in the mutant genotype (35.37%). There was no statistical difference between genotypes (table 3.6., 3.8. & & appendix-6;A.6.1).

For details of the development of non-neutropenic fever in relation to chemotherapy (appendix-6; A.6.2. & A.6.3.).

3.6.6.3.   Analysis of patients developing either febrile (neutropenic or non-neutropenic) event:

There was no statistical significance in the proportion of patients who developed any febrile event either in the presence or absence of neutropenia. There was 56 (45.90%) patients in the wild type, 30 (45.25%) in the heterozygote and 7 (63.64%) in the homozygote genotypes (table 3.6. & appendix-6;A.6.1.).

3.6.6.4.   Analysis of patients developing both febrile (neutropenic and non-neutropenic) events:

The number of patients developing febrile events both while they are neutropenic and non-neutropenic during their course of therapy were 33 (27.05%) in the wild type group and 17 (20.73%) in the mutant variant (heterozygote 16 (22.54%) and in the homozygote 1 (9.09%)), p=>0.05 (table 3.5. & appendix-6;A.6.1.).

Table 3.7.   Number of febrile neutropenic events (FNE) by different chitotriosidase genotypes. The p value calculated by t-test.

Total febrile neutropenic events = 202	Wild type	Heterozygote	Homozygote
Number of febrile neutropenic events. P (wild type: mutant)=0.27	126	63	13
Range per patient	0-5	0-7	0-4
Median Number of events/patient Event/episode of neutropenia Event/neutropenic days	 1 0.5 0.077	 1 0.5 0.1	 1 0.5 0.053

Table 3.8.   Number of febrile non-neutropenic events (FNnE) analysed by different chitotriosidase genotypes. The p value calculated by t-test.

Total febrile non-neutropenic events=114	Wild type	Heterozygote	Homozygote
Number of febrile non-neutropenic events. P (wild type: mutant)=0.23	73	38	3
Range per pt	0-6	0-4	0-1
Median; number of events per pt	0	0	0

3.6.7. Analysis of the types of organism cultured

The likelihood of having positive cultures with a particular organism (bacteria, virus or fungus) was analysed according to the number of febrile events, the number of episodes of neutropenia and the number of patients within each genotype.

3.6.7.1. Analysis of organism cultured per febrile events in the presence and/or absence of neutropenia.

Here the number of febrile events in relation to the organism isolated was calculated in order to evaluate organism prevalence, in different chitotriosidase genotypes in the presence and/or absence of neutropenia.

1-   Number of febrile events with positive bacterial culture

Diagnosis of positive bacterial culture was considered if patients grew gram positive or gram negative bacteria from their blood or central line with the presence of fever, with or without neutropenia. If a patient with multiple febrile events grew the same organism within 5 days with or without neutropenia then this was considered as the same infection and not a new febrile event.

Febrile events with positive bacterial cultures occurred more frequently in patients with the mutant genotype. There was a significant difference between the three proportions (wild type, heterozygote and homozygote) regardless of neutropenic state (p=0.031). The heterozygote genotype had the most febrile events (62.38%) with statistical significance (p=0.037). (Table 3.9.1., figure 3.3. & appendix-6 A.6.10.).

A similar pattern observed when comparing positive bacterial cultures during febrile events associated with neutropenia. Statistically significant differences were observed between wild type, heterozygote and homozygote (p=0.015). The heterozygote genotype has the highest rate of events 67% (table 3.9.2., & figure 3.3. appendix-6 A.6.11.).

However febrile events (FNnE) with positive bacterial cultures occurring in the absence of neutropenia were found to be highest among the homozygote genotype 66.67%. There was no significant difference between the wild type and mutant genotypes (p=0.48). (table 3.9.3., figure 3.3. & appendix-6 A.6.12.).

2-   Number of febrile events with the diagnosis of proven or probable invasive fungal disease (IFD)

Invasive fungal infections can be categorised as possible, probable or proven.[183]

Diagnosis of proven invasive fungal disease (IFD) is made by histopathalogical, cytopathalogical or direct microscopic examination of body material and fluids. The diagnosis of probable IFD is made by combination of radiological appearance, cytology, microscopic examination, culture, BAL and two positive Galactamannan. The diagnosis requires host factor, clinical and mycological criterion.[183] Given the low total number of events in this study proven and probable IFD will be considered as one entity.

Possible IFD is diagnosed when host factors and clinical criterion are present with the absence of mycological criterion.[183] Since in this group it is common to have host and clinical criterion possible IFD is analysed under the category of febrile events with negative cultures (see below) analysis as sole entity see appendix-6 A.6.6.

If febrile events in a patient had multiple isolates of the same fungal pathogen this was considered a continuation of the infection and not a new diagnosis.

The number of febrile events with proven or probable diagnosis was too small to give a clear picture (tables 3.9.1., 3.9.2., 3.9.3., figure 3.3., and Appendix-6 A.6.10., A.6.11., A.6.12.).

3-   Number of febrile events due to viral isolates

There were a few documented viral infections as a cause of febrile events possibly due to the difficulty in isolating and identify relevant organisms.

The proportional febrile events with isolated viruses did not statistically differ between the genotypes (tables 3.9.1., 3.9.2., 3.9.3., figure 3.3., and Appendix-6 A.6.10., A.6.11., A.6.12.).

4-   Number of febrile events of unknown origin (culture negative)

Febrile events without positive microbial culture including possible IFD diagnosis were considered in this category.

Culture negative febrile events with (FUO) occurred frequently within the homozygote genotype 68.75%. There were significant differences between the wild type, heterozygote and homozygote genotypes (p=0.029) (table 3.9.1., figure 3.3. & appendix-6 A.6.10.).

Similarly FUO associated with neutropenia were more likely in the homozygote, (77%) (p=0.007). Statistically there were significant differences between the wild type, heterozygote and homozygote genotypes and between the wild type and homozygote (p=0.008) (table 3.9.2, figure 3.3. & appendix-6 A.6.11.).

Febrile non-neutropenic events showed no significant differences between all genotypes (table 3.9.3, figure 3.3. & appendix-6 A.6.12.).

3.6.7.2.   Analysis of organism cultured per episode of neutropenia

Patients who develop neutropenia are prone to develop infections and become febrile. However not all patients develop fever whilst neutropenic. The reason for this is likely to be multifactorial with involvement of innate and adaptive immunity, the regimen of chemotherapy, endogenous colonisation and pathogen exposure. In this section the proportion of episodes of neutropenia during which patients became febrile for each genotype was calculated.

There was a statistically significant difference in the number of neutropenic episodes with fever due to bacteria between the wild type and mutant populations, (wild type: heterozygote: homozygote proportion p= 0.018 and the wild type:

homozygote proportion p= 0.004). There were total of 69 (24.47%) episodes with positive bacterial growth out of the 282 episodes of neutropenia in the wild type. 2, (5.56%) in the homozygote, and (28.15in the heterozygote (table 3.10., figure 3.3. & appendix-6 A.6.9.).

Table 3.9.1. Total febrile events regardless neutropenia in different chitotriosidase genotypes, and the type of identifiable organism if present. The p value by Chi square and Fisher exact tests: compares the wild type: heterozygote: homozygote proportions

Total febrile events=316	Wild type; Total events=199	Mutant; Total events =117	Heterozygote; Total events=101	Homozygote; Total events=16
Total bacterial +ve events Bacterial +ve/febrile event % P= 0.031	101 50.75%	68 58.12%	63 62.38%	5 31.25%
Total fungal +ve events Fungal+ve/febrile event % P = 0.705	5 2.51%	0	0	0
Total viral +ve events Viral +ve/febrile event % P = 0.214	2 1.01%	2 1.71	2 1.98	0
Total culture -ve events Culture –ve / febrile event % P = 0.029	91 45.73%	47 40.17	36 35.64	11 68.75

Table 3.9.2 Total febrile neutropenic events in different chitotriosidase genotypes, and the type of identifiable organism if present. P value by Chi square and Fisher exact tests: compare the wild type: heterozygote: homozygote proportions.

Total febrile neutropenic events = 202	Wild type; FNE=126	Mutant; FNE=76	Heterozygote; FNE=63	Homozygote; FNE=13
Number of bacterial FNE Bacterial rate/FNE % P =0.015	74 58.73%	45 59.21%	42 67%	3 23%
Number of fungal FNE Fungal rate / FNE% P = 0.609	2 1.6%	0	0	0
Number of viral FNE Viral +ve / FNE% P = 0.393	1 0.8%	2 2.63%	2 3.2	0
Number of culture -ve FNE Culture -ve rate / FNE% P = 0.007	49 38.89%	29 38.16%	19 30.16%	10 77%

Table 3.9.3. Total febrile non-neutropenic events in different chitotriosidase genotypes, and the type of identifiable organism if present. P value by Fisher exact tests: compares the wild type: heterozygote: homozygote proportions .

Total febrile non-neutropenic events = 114	Wild type FNnE=73	Mutant FNnE=41	Heterozygote FNnE=38	Homozygote FNnE=3
Number of bacterial FNnE Bacterial rate/FNnE% P = 0.144	27 36.98%	23 56.10%	21 55.26%	2 66.67%
Number of fungal FNnE Fungal rate / FNnE% P =0.585	3 4.11%	0	0	0
Number of viral FNnE Viral +ve / FNnE% P = 1.000	1 1.36%	0	0	0
Number of culture negative FNnE Culture -ve rate / FNnE% P = 0.392	42 57.53%	18 43.90%	17 44.74%	1 33.33%
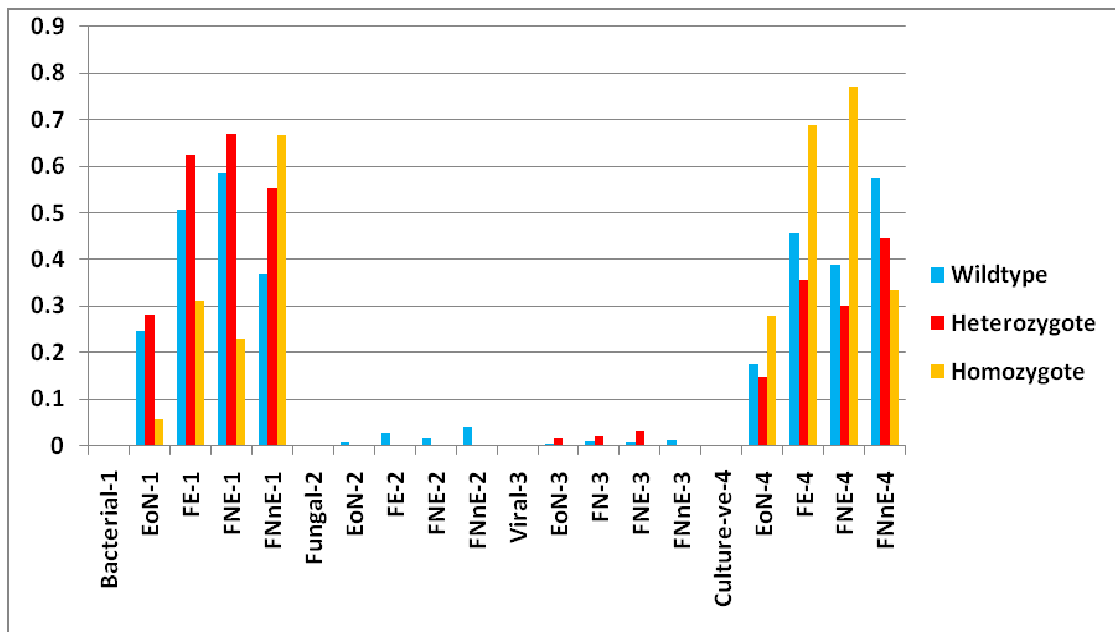
Figure 3.3. Analysis of the types of organism cultured per total events (EoN, FN, FNE and FNnE) in each genotype. (1-Proportion of events with bacterial growth per chitotriosidase genotype, 2-proportion of events with fungal growth per genotype, 3-proportion of events with viral infections per genotype, 4-proportion events with unidentified organism per genotype) (proportion is a 100 of percentage).

Table 3.10. Total episodes of neutropenia associated with febrile events in different chitotriosidase genotypes, and the type of identifiable organism if present. P values by Fisher exact tests compare the wild type: heterozygote: homozygote proportions.

Episodes of neutropenia with febrile events.	Wild type; Total EoN =282	Mutant; Total EoN =171	Heterozygote; Total EoN =135	Homozygote; Total EoN =36
EoN with bacterial FNE. (%) P value = 0.018	69 (24.47%)	40 (23.39%)	38 (28.15%)	2 (5.56%)
EoN with probable/ proven fungal FNE. (%). P value= 1.000	2 (0.71%)	0	0	0
EoN with viral FNE. (%). P value= 0.412	1 (0.35%)	2 (1.17%)	2 (1.46%)	0
EoN with culture negative FNE. (%). P value= 0.190	49 (17.38%)	30 (17.54%)	20 (14.81%)	10 (27.78%)

There was no significant difference between the total episodes of neutropenia due to proven and probable IFD, viral infection or culture negative events (table 3.10., figure 3.3. & appendix-6 A 6.9.).

3.6.7.3. Analysis of organism cultured per patient

In this section all patients developing febrile events were categorised according to the organism grown in their cultures. This analysis was carried out in the presence and/or absence of neutropenia.

1.   Patients with febrile events and cultures positive for bacteria:

There were 38 wild type (31.15%) patients with febrile neutropenic events due to bacterial growth in the cultures and 20 mutant genotype patients (24.39%). The heterozygote variant accounted for 18 events (25.35%) while the homozygote variant for the other 2 events (18.18%). There were no statistically significant differences in the proportions of patients have positive bacterial cultures either when neutropenic or not neutropenic by any genotype (table 3.11.1., figure 3.4. & appendix-6; A.6.4.).

2.   Number of patients with febrile events due to fungal pathogens:

There were too few patients with the diagnosis of proven or probable IFD (and all within the wild type group) for full analysis. There were 2 (1.64%) patients with neutropenic febrile events and 3 (2.46%) patients in the non-neutropenic events.

5 (4.10 %) patients had either neutropenic or non-neutropenic febrile events, while none had both febrile events with the diagnosis of proven or probable IFD, p=ns (table 3.11.2., figure 3.4. & appendix-6 A.6.5.).

There were no statistically significant differences in the proportions of patients from each genotype having febrile neutropenic or non-neutropenic events attributed to a possible fungal infection, see appendix-6 A.6.6.

3.   Number of patients with febrile events due to viruses:

There number of patients with documented viral infections as a cause of febrile events was small. The proportional of patients with isolated viruses as a cause of fever did not statistically differ between the genotypes (table 3.11.3., figure 3.4. & appendix-6 A.6.7.).

Table 3.11.1.   Patients with febrile events and positive bacterial cultures. FE in the presence or absence of neutropenia, in either or both, in different chitotriosidase genotypes. P values by Fisher exact test compare the wild type: heterozygote: homozygote proportions.

Fever and positive bacterial cultures	Wild type; Total =122	Mutant Total=82	Heterozygote Total =71	Homozygote Total =11
Patients with only neutropenic events. (%). P = 0.564	38 (31.15%)	20 (24.39%)	18 (25.35%)	2 (18.18%)
Patients with only non-neutropenic events. (%). P =0.662	13 (10.66%)	10 (12.20%)	8 (11.27%)	2 (18.18%)
Patients with events either neutropenia or non-neutropenic (%). P =0.779	60 (49.18%)	38 (46.34%)	34 (47.89%)	4 (36.36%)
Patients with events, both whilst neutropenic & non-neutropenic (%). P = 0.503	9 (7.38%)	8 (9.76%)	8 (11.27%)	0

Table 3.11.2. Patients with febrile events and proven or probable IFD. FE whilst neutropenic, non-neutropenic, either or both in different chitotriosidase genotypes. P values by Fisher exact test.: compare the wild type: heterozygote: homozygote proportions

Fever with proven and probable IFD	Wild type; Total=122	Mutant Total=82	Heterozygote Total =71	Homozygote Total =11
Pts with only neutropenic events. (%). P= 0.582	2 (1.64%)	0	0	0
Pts with only non-neutropenic events. (%). P =0.618	3 (2.46%)	0	0	0
Pts with total events either neutropenia or non-neutropenia (%). P = 0.283	5 (4.10%)	0	0	0
Pts with events, both whilst neutropenia & non-neutropenia.	0	0	0	0

Table 3.11.3. Patients with febrile events due to viral pathogens. FE whilst neutropenic, non-neutropenic, either or both in different chitotriosidase genotypes. P values by Fisher exact test.: compare the wild type: heterozygote: homozygote proportions

Fever and viral positive event	Wild type; Total=122	Mutant Total=82	Heterozygote Total =71	Homozygote Total =11
Pts with only neutropenic events. (%). P= 0.624	1 (0.82%)	2 (2.44%)	2 (2.82%)	0
Pts with only non-neutropenic events. (%). P = 1.000	1 (0.82%)	0	0	0
Pts with total events either neutropenia or non-neutropenia. (%). P = 0.701	2 (1.64%)	2 (2.44%)	2 (2.82%)	0
Pts with fever, both whilst neutropenia and non-neutropenia	0	0	0	0

Table 3.11.4. Patients with febrile events without identifiable organism. FE whilst neutropenic, non-neutropenic, either or both in different chitotriosidase genotypes. . P values by Fisher exact test.: compare the wild type: heterozygote: homozygote proportions

Fever and culture -ve event	Wild type; Total=122	Mutant Total=82	Heterozygote Total pts=71	Homozygote Total =11
Pts with only neutropenic events. (%). P= 0.068	27 (23.13%)	14 (17.07%)	10 (14.08%)	4 (36.36%)
Pts with only non-neutropenic events. (%). P= 0.243	18 (14.75%)	10 (12.20%)	9 (12.68%)	1 (9.90%)
Pts with total events either neutropenia or non-neutropenia. (%). P= 0.058	59 (48.36%)	27 (32.93%)	22 (30.99%)	5 (45.45%)
Pts with fever, both whilst neutropenia and non-neutropenia. (%). P= 0.214	13 (10.66%)	3 (3.66%)	3 (4.23%)	0 0

4.   Patients with fever with no identified microorganism:

Patients with febrile events without positive microbial culture including possible IFD diagnosis were considered in this category.

The number of patients with only febrile neutropenic events and negative cultures was higher in the homozygote genotype. In comparing the wild type, heterozygote and homozygote, there was a trend towards significance, p=0.068.

A significantly lower number of heterozygote patients had negative cultures than wild type in either neutropenic or non-neutropenic (p=0.018), see appendix-6 A.6.8.

Patients with mutant chitotriosidase seemed not to exhibit any organism more than the wild type (this could be attributed to the small number of patients with documented diagnosis of fungal and viral infections). In contrast unidentified cause of fever (culture negative and possible IFD) was more prevalent in the wild type.

3.6.8.   Course and outcome of the febrile events in relation to chitotriosidase gene status

The outcome of febrile events in different genotypes was analysed, in the presence and absence of neutropenia. In the 86 patients carrying the mutant genotype there were 76 febrile neutropenic events (FNE). These events comprise 279 febrile neutropenic days, 913 days of antimicrobial treatment with a median of 1 and 5 respectively. Out of the 76 FNE in the mutant genotype 6 FNE necessitate Hickman line removal. All of these along with the number of events and patients ended with recovery, ITU admission and death showed no statistical significant differences in both wild type and mutant genotypes with FNEs (table 3.12.). As in the FNEs, the febrile non-neutropenic events (FNnE) showed no differences in the course and outcome in relation to events or number of patients. (table 3.12.).

Chitotriosidase mutation showed no effect in the duration of febrile events or treatment required. The outcome of such febrile events in the presence or absence of neutropenia showed no significant differences.

Table 3.12. Outcome of febrile events in relation to chitotriosidase gene status. (FNE: febrile neutropenic event, FNnE: febrile non-neutropenic event, R: treatment, HL: Hickman line, pts: patients, ITU: intensive treatment unit). P values calculated by Chi square and Fisher exact tests.

Course & Outcome	FNE= 126:76		FNnE= 73:41	
Genotype	Wild type=122	Mutant=86	Wild type=122	Mutant=86
Total febrile days/genotype P=	551 0.14	279	248 0.44	159
Total days of antimicrobial R P=	1704 0.13	913	818 0.25	463
Number of febrile events necessitated HL removal. P=	12 9.52% of 126 0.190	6 7.89% of 76	5 6.85% of 73 0.084	7 17.07% of 41
Number of febrile events with complete recovery. P=	118 (93.65%) 0.768	73 (96.05%)	66 (90.41%) 0.133	33 (80.49%)
Number of pts with recovered febrile events. P=	68 (55.74%) 0.190	40 (45.51%) of pts	42 (34.43%) 0.239	23 (26.74%) of pts
Number of febrile events ended in ITU. P=	6 (4.76%) 0.235	2 (2.63%)	7 (9.59%) 0.169	6 (14.63%)
Number of pts admitted to ITU P=	6 (4.91%) 0.195	2 (2.33%)	5 (4.10%) 0.215	5 (5.81%)
Number of febrile events ended in death. P= Febrile event : death ITU : death	2 (1.59%) 0.333 2 0	2 (2.63%) 1 1	2 (2.74%) 0.109 0 2	4 (9.76%) 2 2
Number of pts died. P=	2 (1.64%) 0.356	2 (2.34%)	2 (1.64%) 0.195	4 (4.65%)
Median Fever days per patient Antimicrobial R days per patient Antimicrobial R days/fever days	2 9 4.5	1 5 5	0 0 0	0 0 0

3.7.   Discussion:

The chitotriosidase gene activities in infections have suggested its possible role in defence mechanisms. These activities include chitinlytic activity towards cell wall chitin of candida[7] and upregulation activity in bacterial infections.[98]

The genes of all known members of chitinase protein family are located on chromosome 1 at 1q31-32.[106] The frequency of mutation in this study was 40.20% in total, the heterozygote mutation accounted for 34.80% and homozygote for 5.39%. Our figures were similar to previous studies.[106, 107]

Several studies have shown predominance of haematological malignancies in males[52, 184, 185] with either bimodal age of distribution as in leukaemia,[51] steep increase in the incidence with age as in lymphomas[185] or increase over the age of 50 as seen in multiple myelomas.[184] In this study males accounted for 59.31% of the haematological malignancies while females for 40.69%. In relation to chitotriosidase mutation, the incidence of haematological malignancies was 56.10% in males and 43.90% in females carrying the mutation.

Age at the time of diagnosis of haematological malignancy ranged between (18-82 years). The incidence of haematological malignancy almost doubled above 71 years from 9.84% in the wild type to 17.07% in the mutant. It is unclear whether chitotriosidase mutation aggravates the incidence of haematological malignancy in older people or affects the age of presentation. This could be addressed in a multivariate analysis of a larger cohort.

Lymphoma was the most frequent diagnosis in our study. In the patients analysed there was a suggestion of worse out come in patients with the mutant chitotriosidase in terms of patients with refractory disease or a progressive course (mutant 6.10% vs wild type 1.64%).

Neutropenia is a frequent sequel of myelosuppressive chemotherapeutic treatment. The duration of neutropenia is typically 7–10 days with variation as a result of the contribution of different factors such as; nature of the disease, intensity of the chemotherapeutic regimen and patient factors. These factors affect not only the duration but also the incidence of neutropenia.[186] In this study neutropenia showed no statistical significance in occurrence and duration according to chitotriosidase genotype (84.43% and 79.27% in wild type and mutant genotypes respectively).

Infection resulting in fever occurs in malignancy due to disease associated defects affecting the neutrophil functions such as chemotaxis, phagocytosis and bactericidal activity. These defects become more pronounced with the fall of neutrophil count and render patient susceptible to opportunistic pathogens and subsequent morbidity.[79]

The number of febrile events was analysed by genotype, and also by presence and/or absence of neutropenia. The proportion of patients with febrile events did not differ by genotype. Our findings suggest that overall susceptibility to infection resulting in fever is not affected by chitotriosidase genotype. Our original hypothesis was that defects in macrophage function due to chitotriosidase mutation may become more apparent during periods of neutropenia. It may be that longer periods of neutropenia are required to detect this effect.

Studies show that only 10-20% of infections are microbiologically documented with identified pathogens in compromised patients.[187] Pneumonia and pulmonary infiltrates accounts for 15-30% of clinically and/or radiologically documented fevers with the majority of these lacking microbiological identification.[188] A further 50% are considered as fever of unknown origin (FUO)[3, 189] and may include conditions such as neutropenic entercolitis and perirectal infection.[189]

The prevalence of particular classes of microorganism positively detected in febrile patients was analysed with respect to chitotriosidase genotype for total number of febrile events, episodes of neutropenia and total patients.

Fungal (proven or probable) and viral infections were too small in number to analyse fully.

The analysis of febrile events in relation to classes of organism showed more positive bacterial cultures as well as unidentified causes (culture –ve) in patients with mutant chitotriosidase.

Febrile events regardless neutropenia with positive bacterial cultures were more likely to occur in the mutant chitotriosidase genotype, increasingly in the

heterozygote genotype. While culture negative febrile events were at increase occurrence with the homozygote mutation (small group; 11patients). The prevalence of these organisms did raise in the presence of neutropenia for both mutant chitotriosidase genotypes.

This finding could reflect the defects in macrophages cytocidal activity against microorganisms and the LPS upregulation[109, 111], which became more pronounced with the presence of neutropenia.

Innate immunity relies on phagocytic cells for the detection and clearance of any pathogen. The inflammatory response varies with different phagocytic cells. Acute inflammation with bacterial destruction and minimal cytokine production is carried by the neutrophils. While the macrophages are antigen presenting cells causing cytokines production and lead to chronic inflammation.[179]

Chitotriosidase enzyme is mainly synthesised by macrophages and to lesser extent by neutrophilic granulocytes.[98, 99] It is also considered a serum marker of macrophages activation.[109, 114] These may reflect more the macrophages rather than the neutrophils status, and so may explain why neutrophils are less affected by the chitotriosidase activity.

The heterogeneity in findings could be explained by the variety of malignancies and chemotherapy and the affect they exert on the neutrophils function as well as patient factors (age mental status and co-morbid disease).

Overall the chitotriosidase mutation seems not to affect the course or the outcome of febrile events whatever the causative organism is. The duration of febrile event and antimicrobial therapy was no different in the genotypes nor outcome of such febrile events.

3.8.   Conclusions:

Our study suggests that mutation in chitotriosidase seems to have:

No effect on the number of episodes of neutropenia or number of neutropenic days per patient.

No effect on the number of febrile events per patient in the presence or absence of neutropenia.

No effect on the in number of febrile events per episode of neutropenia.

Patients carrying the mutation seem to be more susceptible to bacterial infections during the presence or absence of neutropenia.

Patients with double mutations (homozygote) are more prone to develop febrile events with unidentified pathogens in the presence of neutropenia.

Mutation in chitotriosidase seems to have no effect in overall course and outcome of febrile events.

Chapter 4

NOD2 as genetic determinant of sepsis in

haematological malignancy

4.1.   Introduction

Patients with haematological malignancies are at risk of infections due to defects in the bone marrow. Infections could develop due to either the defective neutrophils function as seen in acute leukaemia[67] or immunological deficits of the underlying malignancy, multiple myeloma[190] is an example. In addition, myelosuppression induced chemotherapy, expose those patients to quantitative neutropenia and the susceptibility of infections at unusual sites and by opportunistic pathogens considered non pathogenic in immunocompetent host.[67] The depth and duration of neutropenia can influence type, frequency and severity of infection as well as response to therapy and outcome.[66] The breakdown of the physical barriers externally (skin) and internally (mucous membrane), the use of steroids, and the necessity for indwelling catheters all as a part of the treatment, furthermore increase the liability to infections in the readily immunosuppressed patients.[191, 192]

Sepsis describes clinical syndromes resulting from systemic inflammatory host response. The pathophysiological concept of sepsis suggests inappropriate immune response with the systemic release of pro-inflammatory and anti-inflammatory mediators leading to systemic inflammatory response and systemic manifestations.[78, 79]

Recent studies explored the genetic role in determining sepsis susceptibility, severity and outcome.[164, 193] A number of genetic polymorphisms (single nucleotide

polymorphisms; SNP) have been identified and were linked to risk of infection, organ dysfunction and mortality.[164]

The first line of mucosal host defence detects invading pathogens through several pattern recognition receptors (PRR). Nucleotide binding oligomerization domain (NOD2) is an intracellular protein receptor that induce innate immunity cascade events in response to microbial pathogens invasion. It is a specific sensor receptor to muramyl dipeptides (MDP), peptidoglycans (PGN) and lipopolysaccharides (LPS), motifs derived from gram positive or gram negative bacteria.[138, 139, 194] In addition NOD2 showed a bactericidal function, this was observed by the direct intracellular killing of Salmonella typhimurium in the intestinal epithelial cells.[195] The bactericidal function was inefficient in patients of CD with the mutant NOD2 compared to wild type.[196] These properties suggest a link between bacterial detection, presentation and elimination. NOD2 is expressed in macrophages,[131] dendritic cells,[144] intestinal epithelial[140] and Paneth cells.[145]

Crohns disease (CD) is a chronic inflammatory bowel disease. CD can affect any part of the intestine from mouth to anus with patchy transmural lesions.[197] Etiologically, it is a multifactorial polygenic disease with genetic association. Three SNPs mutations of NOD2 were identified in 30-50% of CD and 15-20% in healthy controls.[152] The risk of developing CD is 2-4 fold with heterozygote mutation and risk further increases 20-40 folds in individuals with the homozygote mutation.[146, 148, 149] Recently studies provided evidences suggesting the trigger of inflammation in CD is impaired innate immune response to intestinal microbial flora.[95, 198] A mutation in NOD2 leads to an attenuated NF-κB activity and impaired immune response leading to functional impairment of the phagocytic cells, such as the impaired bactericidal function by the intestinal epithelial cells in CD associated with frameshift 3020insC SNP mutation.[195] Other defects in innate immunity seen in CD include neutrophil chemotaxis,[199] respiratory burst,[200] and phagocytosis.[201] The resemblance of CD to some infections such as intestinal tuberculosis suggested the infectious origin of this inflammatory disease, along with the clinical beneficial use of antibiotics.[152]

Evidence of impaired innate immune response by CD patients was observed by Marks et al. Patients with CD were subjected to intradermal injection of killed bacteria and were observed for local and general inflammatory response in comparison to healthy controls. CD patients provoked inadequate innate immune response, this was suggested by the less blood flow, lower cytokines production and reduced neutrophil accumulation at the site of the trauma.[202]

Immunosuppression can render an individual more susceptible to infections as seen in patients with haematological malignancies. Neutrophils in this patient can be inadequate in their function due to the natural process of the disease or low in quantity, chemotherapy induced neutropenia. Patients with neutropenia are readily liable to infections, however not all neutropenic patients develop infections indicating a possible genetic role. The additive effects of inadequate neutrophil function, chemotherapy and genetic variation were the basis of our study.

In this present study we identified the NOD2 status in patients with haematological malignancies and relate the impact of NOD2 mutation to the development of infection and sepsis. Evaluation of sepsis was studied in the same group of patients in relation to absolute neutrophil count (neutropenic or non-neutropenic), the causative organism, and outcome.

4.2. Hypothesis:

Our hypothesis is that patients carrying mutations in NOD2, a gene mediating innate immunity, are at increased risk of infection and sepsis during periods when they are neutropenic.

4.3.   Objectives:

1) To determine the effect of NOD2 status on the incidence and outcome of sepsis in patients with haematological malignancy, including those with leukaemia, lymphoma, and myeloma.

2) To compare the effect of NOD2 status on the incidence and outcome of sepsis in patients with haematological malignancy who are neutropenic and those who are not.

3) To examine the effect of NOD2 status on the prevalence of organisms in neutropenic and non-neutropenic patients with haematological malignancies.

4) Correlate duration of neutropenia with the NOD2 status.

4.4.   Materials and methods:

General material and methods are described in Chapter 2.

Inclusion and exclusion criteria are also described in chapter 2.

Recruitment of patients with haematological malignancy was performed in the Royal Free Hospital.

The study was ethically approved by the central office for research ethics committees (REC Ref: 06/Q0501/56-Date 3/4/06. R&D ID:7414-Date 28/6/08). All patients gave informed consent. They were followed up during their in-patient stay. Clinical data were collected daily by the clinical fellow. Observations were recorded including ANC, temperature, related investigations (microbial and imaging) to determine the source and type of sepsis, and antimicrobial management. Investigation and data of patients recruited from the out-patient department or after patients discharge were recorded electronically or from paper medical records by the clinical research fellow (LS).

The number of patients included in this study was 201 from the 204 patients meeting the inclusion criteria. Exclusion of the three samples was due to low concentration and insufficient amount of DNA. These samples could not be replaced as 2 patients were deceased and one received allogenic BM transplant (BMTx).

DNA was extracted from whole blood. NOD2 identification was carried out initially by gene scanning alternatively and due to technical difficulties this was replaced by pyrosequencing. Detection of SNP8 and SNP12 mutation (insertion) was successful by pyrosequencing. The SNP13 mutation (deletion/frameshift) was not included as the pyrosequencing software was not designed for deletion identification.Another reason and due to time limitation an alternative instituite or labratory could not be considered. Methods in detail are mentioned in chapter 2.

4.5.   Statistics:

The p value was calculated by the Chi square, and by Fisher exact if figures ≤ 10 if comparing two dimensions. The p value of ≤0.05 considered significant. (http://www.physics.csbsju.edu/stats/contingency_NROW_NCOLUMN_form.html) (http://www.physics.csbsju.edu/stats/fisher.form.html).   P   value   differences   were calculated between the wild type and mutant proportion. Mean, median were calculated along with graphs were prepared using Microsoft excel.

4.6.   Results:

4.6.1.   Incidence of NOD2 mutation:

Mutation: Out of the 201 patients, 184 (91.54%) were wild type for NOD2 and 17 (8.46%) were mutant. Of the 17 mutant genotype patients 7 (41.18%) were heterozygote for SNP 8. There was 12 patients with SNP 12 mutation, 11 (64.71%)

heterozygote and 1 (5.88%) homozygote for the mutation. 2 patients exhibited compound heterozygosity for SNP 8 and 12 (table 4.1.).

In this study population, frequency of mutation was 3.48% heterozygosity for SNP8. While SNP12 frequent occurrence was 5.47% heterozygosity and 0.50% homozygosity for the mutation.

4.6.2.　　　Demographic characteristics:

A total of 201 patients were included in this section of the study. Males accounted for 59.20% and females for 40.80%. The age ranged between 18-81 years at the time of diagnosis (table 4.2.). No statistical differences were found in the gender, age or diagnosis distribution between NOD2 genotypes.

Genotype	SNP8 (%)	SNP12 (%)
Heterozygote	7 (3.48)	11 (5.47)
Homozygote	0	1 (0.55)

Table 4.1.
The frequency of NOD2-SNPs mutations In 201 DNA samples, the only homozygote mutation was in SNP 12. (2 patients exhibited compound heterozygosity for SNP 8 and 12).

Table 4.2.　　　Demographic characteristics in the study population. The number of patients and proportion by sex and age at diagnosis of haematological malignancy in relation to NOD2 status. (M: male, F: female). P value calculated by Fisher exact.

NOD2 status	Sex		Age at diagnosis							
	M	F	18-20	21-30	31-40	41-50	51-60	61-70	71-80	81-90
Total;201 %	119 59.20	82 40.80	5 2.50	19 9.45	24 11.94	28 13.93	52 25.87	47 23.38	23 11.44	3 1.49
Wild type; 184 91.54%	111 59.68	73 39.25	5 2.72	17 9.24	23 12.5	26 14.13	46 25	43 23.36	22 11.96	2 1.09
Mutant; 17 8.46%	8 47.06	9 52.94	0 0	2 11.77	1 5.88	2 11.77	6 35.29	4 23.53	1 5.88	1 5.88
P=	Mutation M:F = 0.209		0.699	0.288	0.265	0.288	0.140	0.233	0.279	0.215

Table 4.3. Diagnosis with different haematological malignancies in our study population. The number of patients and frequencies (%) in total in relation to NOD2 status. P value calculated by Fisher exact.

NOD2 status	Diagnosis				
	Lymphoma	Multiple myeloma	Acute Leukaemia	Chronic Leukaemia	Other
Total 201; (%)	106 (52.74%)	32 (15.92%)	50 (24.88%)	11 (5.47%)	2 (0.99%)
Wild type; 184 (91.54%)	97 (52.72%)	30 (16.30%)	47 (25.54%)	8 (4.35%)	2 (1.09%)
Mutant; 17 (8.46%)	9 (52.94%)	2 (11.76%)	3 (17.65%)	3 (17.65%)	0
P=	0.594	0.262	0.193	0.046	0.838
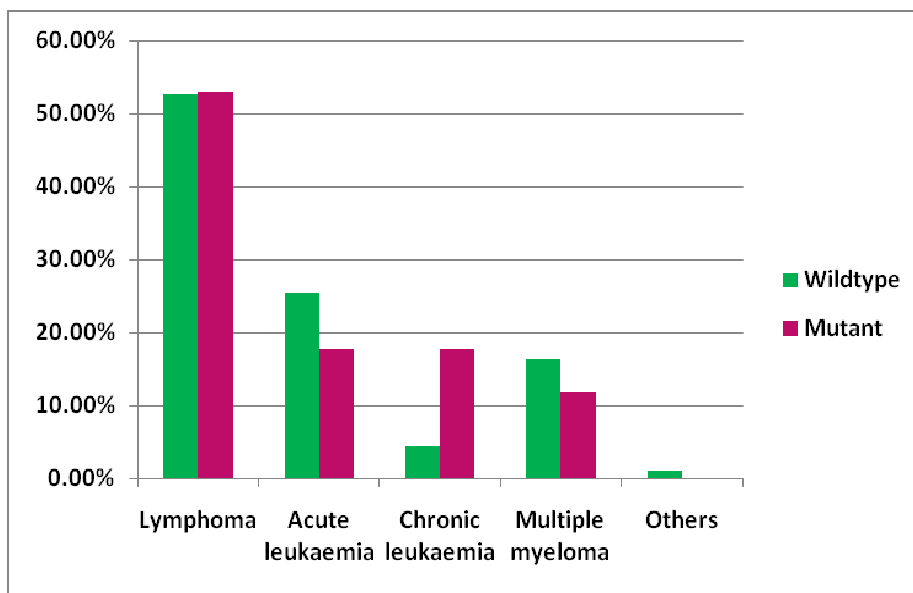
Figure 4.1.

Diagnosis of different haematological malignancies. Frequencies in relation to NOD2 genotype.

4.6.3.   Range of Diagnoses

In this study population the diagnosis of all types of haematological malignancies showed no statistical significance except for chronic (lymphoblastic and myeloid) leukaemia. There were a small number of patients with lymphocytic or myeloid leukaemia and so we gathered all types into acute and chronic leukaemia. NHL accounted for 8 (47.06%) cases while AML (17.65%) accounted for all of acute leukaemia in the mutant NOD2. Chronic leukaemia (lymphoblastic and myeloid)

diagnosis was higher in the mutant NOD2 with statistical significance, p=0.046 (table 4.3. & figure 4.1.).

Table 4.4. Outcome of the patients included in the study in relation to NOD2 gene status. (CR: complete remission, PR: partial remission, Allo: allogenic transplant, On R: still on treatment at last follow up). P value calculated by Fisher exact.

NOD2 status	Outcome						
	CR	PR	Allo	Death	On R	Relapse	Progressive
Total 201; (%)	60 29.85%	42 20.90%	32 15.92%	34 16.91%	17 8.46%	9 4.48%	7 3.48%
Wild type; 184 (91.54%)	54 29.35%	40 21.74%	29 15.76%	33 17.94%	14 7.61%	7 3.80%	7 3.80%
Mutant; 17 (8.46%)	6 35.29%	2 11.76%	3 17.65%	1 5.88%	3 17.65%	2 11.76%	0 0
P=	0.184	0.175	0.245	0.142	0.120	0.140	0.533
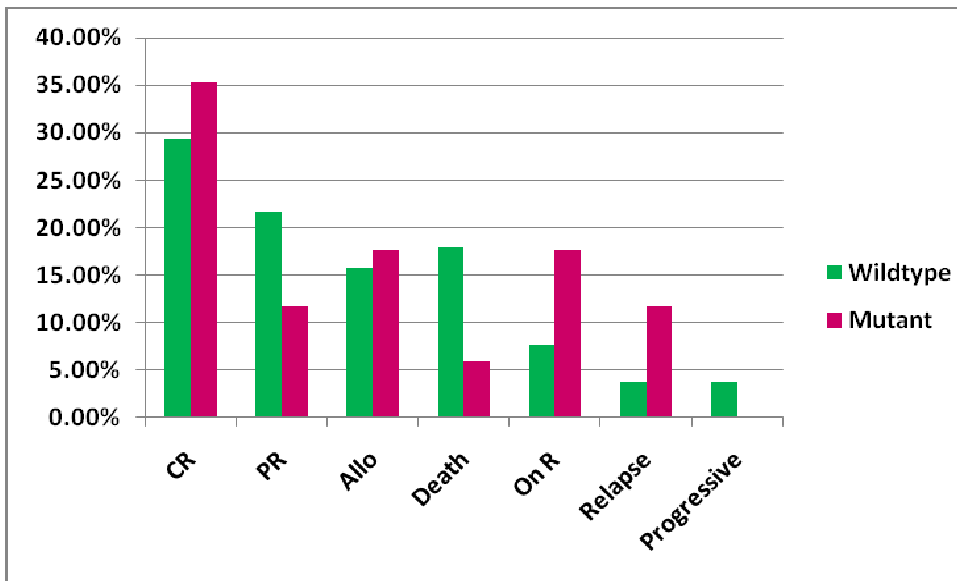
Figure 4.2.

Outcome of haematological malignancies in the study population in %, in relation to NOD2 genotype.

4.6.4. Outcome of the haematological malignancy in relation to NOD2 status

Out of the total 201 patients, 29.85% entered complete remission, 15.92% ended receiving allogenic BMTx and 4.48% relapsed while the study was in progress. The different outcomes did not show any significant differences between NOD2

genotypes. CR, allogenic transplant and relapse, p=0.184, 0.245 and 0.140 respectively (table 4.4. & figure 4.2.).

4.6.5.   Analysis of episodes of neutropenia (EoN):

Neutropenia is known to occur with haematological malignancies, and additionally is common side effect of chemotherapy. Absolute neutrophil count of 0.5×10^9/L and duration longer than (7-10 days) increases the patient liability to infections. In this section neutropenia was analysed according to; incidence and duration for each genotype. We also looked into the number of patients per genotype developing neutropenia and the frequency of neutropenic episodes in these patients.

4.6.5.1.   Number of patients with neutropenic episode per genotype:

The total number of patients developing episodes of neutropenia in the wild type was 153 out of 184 patients (83.15%) whilst in the mutant genotype 12 out of the 17 patients (70.59%) became neutropenic during their treatment. There were no statistically significant differences between the NOD2 genotypes, (table 4.5.).

4.6.5.2.   Number of neutropenic episodes per genotype:

There were 450 episodes of neutropenia in total, 419 episodes out of the 450 occurred in patients carrying the wild type NOD2, while 31 out of the 450 in the mutant genotype patients. There was no statistical difference in the proportion of each genotype exhibiting episodes of neutropenia (table 4.6.).

4.6.5.3.   Duration of neutropenic episode:

The duration of neutropenic episodes varied. We calculated the total number of days of neutropenia for each genotype, to evaluate if NOD2 mutation affected the time to neutrophil regeneration. The total neutropenic days per genotype were as follow, wild type 4006 and mutant genotype 262 with no statistical differences (table 4.6.). Although range of neutropenic days per patient was longer in the wild

type the median of neutropenic days per patient and the median of neutropenic days per episode did not differ between NOD2 genotypes (table 4.6.).

Total patients = 201	Wild type = 184	Mutant = 17
Total patients (%)	(91.54%)	(8.46%)
Pts with EoN in each genotype (p= 0.196)	153 (83.15%)	12 (70.59%)

Table 4.5.
Total number of patients (Pts) and total patients with episodes of neutropenia (EoN) in each NOD2 genotype. % of neutropenic patients within each genotype. P value calculated by Chi square

Total episodes of neutropenia = 450	Wild type	Mutant
Total pts / genotype	184	17
Total neutropenic episodes P (wild type: mutant)=0.16	419	31
Total neutropenic days P (wild type: mutant)=0.12	4006	262
Range Episodes of neutropenia / pt Neutropenic days per patient	0-13 0-218	0-6 0-78
Median Episodes of neutropenia/patient Neutropenic days/patient Day/episode	2 10 5	2 10 5

Table 4.6.
Episodes of neutropenia (EoN) analysed by different NOD2 genotypes. P value calculated by t-test.

4.6.6.   Analysis of febrile events:

Fever of 38.5°C on one occasion or fever of 38°C on two occasions was diagnosed as infective febrile event and is a call for initiation of antibiotic cover.

Occurrence of infection was analyzed in this section to determine if NOD2 mutation has any effect on such event occurrences. First we analysed the incidence of febrile events per genotype then the number of patients in each genotype who development such febrile events in the presence and/or absence of neutropenia.

4.6.6.1   <u>Febrile neutropenic events (FNE):</u>

The total number of febrile events was 309; 281 febrile events occurred during neutropenia while 28 occurred in the absence of neutropenia (table 4.7.).

There were no statistical differences in the number of patients developing febrile events, neutropenic, non-neutropenic, or both for all NOD2 genotypes (table 4.7.).

The number of febrile neutropenic event per episode or neutropenic days showed no statistical differences and was the same in all NOD2 genotypes (table 4.8.).

4.6.6.2.   Febrile non-neutropenic events (FNnE):

The number of patients with febrile events not associated with neutropenia was 69 out of the 184 in the wild type (37.5%) and 6 out of the 17 in the mutant genotype (35.29%). There was no statistical difference between NOD2 genotypes (table 4.7.).

The number of febrile neutropenic event per episode or neutropenic days showed no statistical differences and was the same in NOD2 genotypes (table 4.9.).

4.6.6.3.   Analysis of patients developing either febrile event:

There was no statistical significance in the number of patients who developed a febrile event either with the presence or absence of neutropenia in NOD2 genotypes, (table 4.7.).

4.6.6.4.   Analysis of patients developing both febrile events:

The Number of patients developing febrile events both while they are neutropenic and non-neutropenic during their course of therapy showed no significant statistical differences (table 4.7.).

4.6.7.   Analysis of the types of organism cultured

The likelihood of having positive cultures with a particular organism (bacteria, virus, fungus or unidentified organism) was analysed according to the number of febrile

events, the number of episodes of neutropenia and the number of patients within each genotype.

Total	Wild type	Mutant
Total pt 201 (%)	184 (91.54%)	17 (8.46%)
Total Febrile events 309 (%;events genotype/total event)	281 (90.94%)	28 (9.06%)
Total 196 FNE (%;events genotype/total event)	178 (90.82%)	18 (9.18%)
Total 113 FNnE (%;events genotype/total event)	103 (91.15%)	10 (8.85%)
Pts with FNE (% pts of FNE/ genotype pts). P = 0.436	101 (54.89%)	11 (64.71%)
Pts with FNnE (% pts of FNnE/ genotype pts). P = 0.857	69 (37.5%)	6 (35.29%)
Pts with either febrile events P = 0.723	84 (45.65%)	7 (41.18%)
Pts with both febrile events. P = 0.613	44 (23.91%)	5 (29.41%)

Table 4.7.
Total febrile events in each genotype. Proportions of patients (Pts) out of each NOD2genotype with febrile events whilst neutropenic (FNE), non-neutropenic (FNnE), either (FNE or FNnE) or both febrile events (FNE and FNnE). P value calculated by the Chi square or Fisher exact test when figures are $\leq$ 10, P value compares the wild type: mutant proportions.

Total febrile neutropenic events = 196	Wild type	Mutant
Number of febrile neutropenic events. P (wild type: mutant)=0.38	178	18
Range per patient	0-7	0-4
Median Number of events/patient Event/episode of neutropenia Event/neutropenic days	1 0.5 0.1	1 0.5 0.1

Table 4.8.
Number of febrile neutropenic events (FNE) analysed by different NOD2 genotypes. P value calculated by t-test.

Total febrile non-neutropenic events=113	Wild type	Mutant
Number of febrile non-neutropenic events P (wild type: mutant)=0.45	103	10
Range per pt	0-6	0-2
Median; Number of events per pt	0	0

Table 4.9.
Number of febrile non-neutropenic events (FNnE) analysed by different NOD2 genotypes. P value calculated by t-test.

4.6.7.1. Analysis of organism cultured per febrile events in the presence and/or absence of neutropenia.

Here we calculate the number of febrile events in relation to isolated organism, in order to evaluate organism prevalence in NOD2 genotypes in the presence and/or absence of neutropenia.

1- Number of febrile events with positive bacterial culture

Diagnosis of positive bacterial culture was considered if patients grew positive or gram negative bacteria from their blood or central line with the presence of fever, with or without neutropenia. If a patient with multiple febrile events grew the same organism within 5 days with or without neutropenia then this was considered as the same infection and not a new febrile event.

Febrile events with positive bacterial culture occurred in frequency of 80% in the mutant genotype compared to 40.78% in the wild type NOD2, these differences were highly significant in the absence of neutropenia (p=0.017). While this was not the case in febrile events associated with neutropenia or regardless and positive bacterial growth (table 4.10.1., 4.10.2., 4.10.3.).

2- Number of febrile events with the diagnosis of proven or probable invasive fungal disease (IFD)

Invasive fungal infections can be categorised as possible, probable or proven.[183] Diagnosis of proven IFD is made by histopathalogical, cytopathalogical or direct microscopic examination of body material and fluids. The diagnosis of probable IFD is made by combination of radiological appearance, cytology, microscopic examination, culture, BAL and two positive Galactamannan. The diagnosis requires host factor, clinical and mycological criterion.[183] Given the low total number of events in this study proven and probable IFD will be considered as one entity.

Possible IFD is diagnosed when host factors and clinical criterion are present with the absence of mycological criterion.[183] Since in this group it is common to have

host and clinical criterion possible IFD is analysed under the category of febrile events with negative cultures. If febrile events in a patient had multiple isolates of the same fungal pathogen this was considered a continuation of the infection and not a new diagnosis.

The number of febrile events with proven or probable diagnosis was too small to give a clear picture (tables 4.10.1., 4.10.2., 4.10.3.).

3-   Number of febrile events due to viral isolates

There were a few documented viral infections as a cause of febrile events possibly due to the difficulty in isolating and identify relevant organisms.

The proportional febrile events regardless neutropenia with isolated viruses showed a trend (p=0.061) with occurrence of 7.14% in the mutant genotype compared to 1.07% in the wild type NOD2 genotype (tables 4.10.1., 4.10.2., 4.10.3.).

4-   Number of febrile events of unknown origin (culture negative)

Febrile events without positive microbial culture including possible IFD diagnosis were considered in this category.

There were statistical differences (p=0.029) in the febrile events in the absence of neutropenia with unidentified organism between NOD2 genotypes. Such events occurred in frequency of 20% and 55.34% in NOD2 mutant and wild type respectively (table 4.10.1, table 4.10.2, table 4.10.3)

Mutant NOD2 genotype and febrile events with the absence of neutropenia showed statistical significance. These events were related to mutant genotype in opposite way. Infection due to bacterial growth was high in incidence in the mutant NOD2, while fever with unidentified organism was lower in occurrence in the same genotype (table 4.10.3.).

A trend of significance was also shown in the mutant genotype in febrile events regardless neutropenia and viral isolates (table 4.10.1.).

Total febrile events=309	Wild type; Total events=281	Mutant; Total events =28
Total bact +ve events Bacterial +ve/febrile event % P value 0.396	147 52.31%	17 60.71%
Total fung +ve events Fungal+ve/febrile event % P value 0.620	5 1.78%	0
Total viral +ve events Viral +ve/febrile event % P value 0.061	3 1.07%	2 7.14%
Total culture -ve events Culture –ve / febrile event % P value 0.196	126 44.84%	9 32.14%

Table 4.10.1.
Total febrile events regardless neutropenia in different NOD2 genotypes, and the type of identifiable organism if present. P value compare the wild type: mutant proportion. P value calculated by Chi square or Fisher exact for figures $\leq$ 10.

Total febrile neutropenic events =196	Wild type; FNE=178	Mutant; FNE=18
Number of bact FNE Bacterial rate/FNE % P value 0.461	105 59.55%	9 50%
Number of fung FNE Fungal rate / FNE% P value 0.824	2 1.12%	0
Number of viral FNE Viral +ve / FNE% P value 0.229	2 1.12%	1 5.56%
Number of culture -ve FNE Culture -ve rate / FNE% P value 0.992	69 38.76%	7 38.89%

Table 4.10.2.
Total febrile neutropenic events in different NOD2 genotypes, and the type of identifiable organism if present. P value calculated by Chi square or Fisher exact for figures $\leq$ 10, compares the wild type: mutant proportion.

Total febrile non-neutropenic events =113	Wild type FNnE=103	Mutant FNnE=10
Number of bact FNnE Bacterial rate/FNnE% P value 0.017	42 40.78%	8 80%
Number of fung FNnE Fungal rate / FNnE% P value 0.830	2 1.94%	0
Number of viral FNnE Viral +ve / FNnE% P value 0.912	1 0.97%	0
Number of culture negative FNnE Culture -ve rate / FNnE% P value 0.029	57 55.34%	2 20%

Table 4.10.3.
Total febrile non-neutropenic events in different NOD2 genotypes, and the type of identifiable organism if present. P value calculated by Chi square or Fisher exact for figures $\leq$ 10, compares the wild type: mutant proportion.

Episodes of neutropenia with febrile events.	Wild type; Total EoN=419	Mutant; Total EoN=31
EoN with bacterial FNE. (%) P = 0.128	98 (23.39%)	9 (29.03%)
EoN with probable/ proven fungal FNE. (%). P= 0.867	2 (0.48%)	0
EoN with viral FNE. (%). P v= 0.180	2 (0.48%)	1 (3.22%)
EoN with culture negative FNE. (%). P v= 0.128	70 (16.71%)	7 (22.58%)

Table 4.11. Total episodes of neutropenia associated with febrile events in different NOD2 genotypes, and the type of identifiable organism if present. P values by Fisher exact tests compare the wild type: mutant proportions.

4.6.7.2. Analysis of organism cultured per episode of neutropenia

Patients with neutropenia can easily become febrile with the exposure to different organisms and the development of infections.

In this section the proportion of episodes of neutropenia during which patients became febrile for each genotype was calculated.

There were no statistical differences in the number of neutropenic episodes with fever due to any organism between NOD2 genotypes. All infections with pathogens such as bacteria, virus, fungal or even when cultures were negative to any growth were considered in this section (table 4.11., figure 4.3.).

4.6.7.3. Analysis of organism cultured per patient

In this section all patients developing febrile events were categorised according to the organism grown in their cultures. This analysis was carried out in the presence and/or absence of neutropenia.

Number of patients with febrile event and identified or unidentified cause of such events showed no statistical differences in NOD2 genotypes in the presence of neutropenia.

A trend of significance was seen in fever with positive bacterial culture in the absence of neutropenia in patients carrying the mutant genotype (table 4.12.1, 4.12.2., 4.12.3., 4.12.4.).
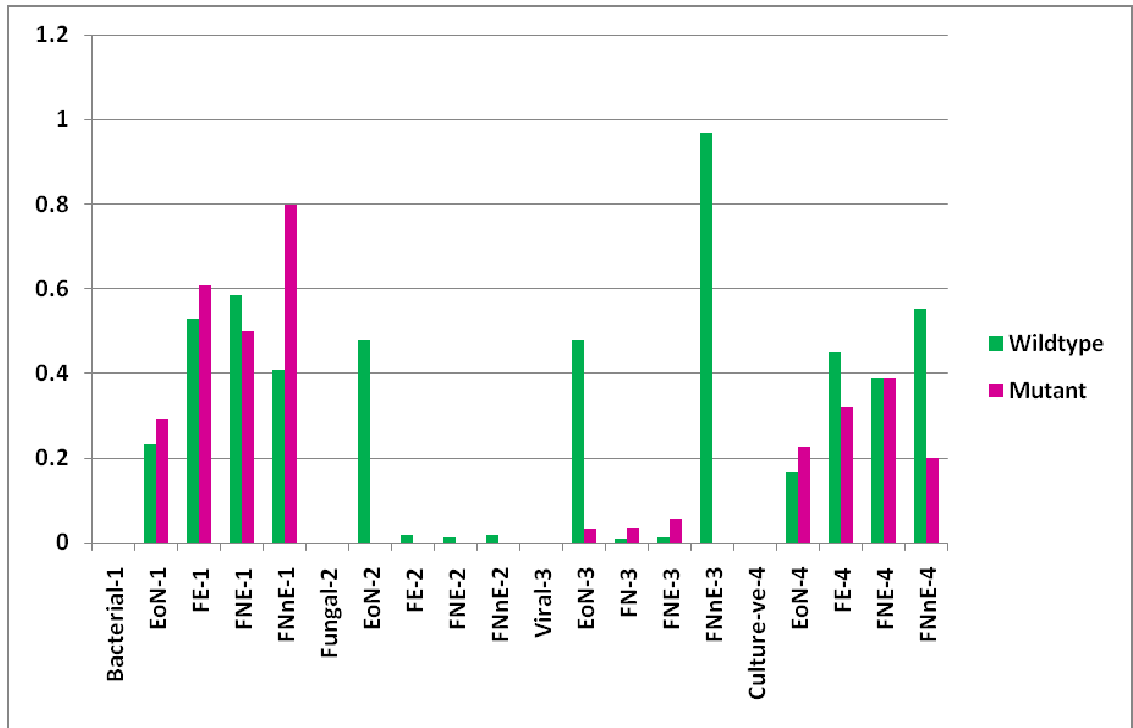
Figure 4.3. Analysis of the types of organism cultured per total events (EoN, FN, FNE and FNnE) in each genotype. (1-Proportion of events with bacterial growth per NOD2 genotype, 2-proportion of events with fungal growth per genotype, 3-proportion of events with viral infections per genotype, 4-proportion events with unidentified organism per genotype) (proportion is a 100 of percentage).

Fever and positive bacterial cultures	Wild type; Total pts= 184	Mutant; Total pts= 17
Patients with only neutropenic events. (%). P = 0.212	52 (28.26%)	4 (23.53%)
Patients with only non-neutropenic events. (%). P =0.082	19 (10.33%)	4 (23.53%)
Patients with events either neutropenia or non-neutropenic (%). P =0.00	86 (46.74%)	0
Patients with events, both whilst neutropenic & non-neutropenic (%). P = 0.271	15 (8.15%)	2 (11.76%)

Table 4.12.1. Patients with febrile events and positive bacterial cultures. FE in the presence or absence of neutropenia, in either or both, in different NOD2 genotypes. P values by Fisher exact test compare the wild type: mutant proportions.

Fever with proven and probable IFD	Wild type; Total pts= 184	Mutant; Total pts= 17
Pts with only neutropenic events. (%). P= 0.838	2 (1.09%)	0
Pts with only non-neutropenic events. (%). P =0.766	3 (1.63%)	0
Pts with total events either neutropenia or non-neutropenia (%). P = 0.640	5 (2.72%)	0
Pts with events, both whilst neutropenia & non-neutropenia.	0	0

Table 4.12.2. Patients with febrile events and proven or probable IFD. FE whilst neutropenic, non-neutropenic, either or both in different NOD2genotypes. P values by Fisher exact test compare the wild type :mutant proportions

Fever and viral positive event	Wild type; Total pts= 184	Mutant; Total pts= 17
Pts with only neutropenic events. (%). P= 0.215	2 (1.09%)	1 (5.88%)
Pts with only non-neutropenic events. (%). P = 0.915	1	0
Pts with total events either neutropenia or non-neutropenia. (%). P = 0.263	3 (1.63%)	1 (5.88%)
Pts with fever, both whilst neutropenia & non-neutropenia	0	0

Table 4.12.3. Patients with febrile events due to viral pathogens. FE whilst neutropenic, non-neutropenic, either or both in different NOD2 genotypes. P values by Fisher exact test compare the wild type :mutant proportions

Fever and culture -ve event	Wild type; Total pts= 184	Mutant; Total pts= 17
Pts with only neutropenic events. (%) P= 0.249	37 (20.11%)	3 (17.65%)
Pts with only non-neutropenic events. (%). P= 0.211	27 (14.67%)	1 (5.88%)
Pts with total events either neutropenia or non-neutropenia. (%) P= 0.118	79 (42.93%)	5 (29.41%)
Pts with fever, both whilst neutropenia & non-neutropenia. (%). P= 0.379	14 (7.61%)	1 (5.88%)

Table 4.12.4. Patients with febrile events without identifiable organism. FE whilst neutropenic, non-neutropenic, either or both in different chitotriosidase genotypes. . P values by Fisher exact test compare the wild type : mutant proportions

Table 4.13. Outcome of febrile events in relation to NOD2 gene status. (FNE: febrile neutropenic event, FNnE: febrile non-neutropenic event, R: treatment, HL: Hickman line, pts: patients, ITU: intensive treatment unit). P values calculated by Fisher exact and t-tests.

Course & Outcome	FNE= 686:102		FNnE= 103:10	
Genotype	Wild type= 184	Mutant= 17	Wild type= 184	Mutant= 17
Total febrile days/genotype P=	686 0.191 (t-test)	102	368 0.500	34
Total days of antimicrobial R P=	2269 0.294	257	1157 0.475	104
Number of febrile events necessitated HL removal. P=	15 8.43% of 178 0.284 (Fisher)	2 11.11% of 18	9 8.74% of 103 0.061	3 30% of 10
Number of febrile events with complete recovery. P=	169 (94.94%) 0.267	16 (88.89%)	89 (86.41%) 0.606	9 (90%)
Number of pts with recovered febrile events. P=	95 (51.63%) 0.378	10 (58.82%)	59 (32.06%) 0.212	5 (29.41%)
Number of febrile events ended in ITU. P=	6 (3.37%) 0.133	2 (11.11%)	12 (11.65%) 0.398	1 (10%)
Number of pts admitted to ITU P=	6 (3.26%) 0.118	2 (11.76%)	5 (2.72%) 0.333	1 (5.88%)
Number of febrile events ended in death. P= Febrile event : death ITU : death	4 (2.25%) 0.678 3 1	0	6 (5.82%) 0.566 3 3	0
Number of pts died. P=	4 (2.17%) 0.700	0	6 (3.26%) 0.584	0
Median Fever days per patient Antimicrobial R days per patient Antimicrobial R days/fever days	1 7 7	1 10 10	0 0 0	0 0 0

4.6.8. Course and outcome of the febrile events in relation to NOD2 gene status

The outcome of febrile events in different genotypes was analysed, in the presence and absence of neutropenia. In the 17 patients carrying the mutant genotype there were 18 febrile neutropenic events (FNE). These events comprise 102 febrile neutropenic days, 257 days of antimicrobial treatment with a median of 1 and 10 respectively. Out of the 18 FNE in the mutant genotype 2 FNE necessitate Hickman line removal. All of these along with the number of events and patients

ended with recovery, ITU admission and death showed no statistical significant differences in both wild type and mutant genotypes with FNEs (table 4.13.).

The febrile non-neutropenic events (FNnE) necessitated Hickman line removal were higher in the mutant NOD2 (30%) compared to wild type (8.74%). These differences showed a trend of significance, p=0.061.

Otherwise as in the FNE, the febrile non-neutropenic events (FNnE) showed no differences in the course and outcome in relation to events or number of patients (table 4.13.).

NOD2 mutation showed no effect in the duration of febrile events or treatment required. The outcome of such febrile events showed no significant differences in the presence of neutropenia.

 The same outcome was seen in the absence of neutropenia, except with the number of febrile events necessitated Hickman removal as a part of management in the mutant NOD2.

4.7.　　　Discussion

The cytoplasmic localization of NOD2, provides the cellular compartment protection against invasive bacterial organisms by a variety of mechanisms.[134] Three major polymorphisms (L1007fsinsC, Arg702Trp and Gly908Arg) within the NOD2-LRR region have been identified and genetically associated with CD in both European and American populations.[4, 5, 130, 149]

In this study the aim was to look in the three major variants of NOD2 in relation to sepsis. The frameshift L1007fsins (SNP13) was eventually not included due to technical difficulties; software designed only for missense detection not the frameshift mutation.

The incidence of the NOD2 mutation had been investigated by different groups in relation to ethnic and geographical factors. The mutation rate was highest in

Belgium and Canada and lowest in Finnish populations. While it was absent in Japanese, Korean and Han Chinese populations.[147] NOD2 mutation have been of interest to investigators relating frequency of mutations in general population and CD.[130, 149]

The incidence of mutation in our study population (201 patients of multiethnic background), was 8.46%. The heterozygote state accounted for 3.48% and 5.47% for SNP8 and SNP12 respectively. Homozygote mutations accounted for (0.50%), and that occurrence was in SNP12. While compound heterozygosity for both SNPs occurred at rate of 0.99% in our study population.

This finding falls between the frequency of NOD2 mutations found by various investigators.[130, 146, 149, 152] the incidence of Arg702Trp (SNP8) varies between 4.5[147]-2.7[151] and Gly908Arg (SNP12) was 5.5[203]-0.6[149], which is compatible with our finding.

Several studies have looked into occurrence of malignancy and NOD2 mutation, but most looked in the frameshift L1007fsinsC variant (SNP13). Malignancies such as colorectal,[204, 205] breast,[206, 207] and NHL[208] were all associated with L1007fsinsC. While gastric lymphoma in H pylori infected patients was associated with the three major polymorphisms of NOD2.[209] In this study NOD2 mutation (Arg702Trp (SNP8) and Gly908Arg (SNP12)) showed a significant association with chronic leukaemia (all types of chronic leukaemia). There were 17.65% (three cases) in the NOD2 mutation genotype compared to 4.35% in the wild type genotype in our study population. The chronic leukaemia patients were all mutant for SNP8, and one was carrying double allele for SNP8 and SNP12. This association with Arg702Trp (SNP8) requires verification in a larger based study.

There was no impact on the diagnosis of haematological malignancies in relation to sex or age distribution. The NOD2 mutation also did not seem to affect the course and outcome of haematological malignancy.

Bone marrow suppression can readily occur as a result of the haematological malignancy itself , as well as the myelosuppressive chemotherapeutic effect.[210]

Neutropenia, the fall of absolute neutrophil count, ANC ≤ 500 cell/mm^3, is a frequent side effect of chemotherapy. Neutropenia as well as the duration of neutropenia (≥7-10 days) are important parameters for infection severity, frequency, treatment and outcome.[210, 211] In this study neutropenia showed no significant differences in occurrence and duration according to NOD2 genotypes.

In recent years the role of genetic factors determining the susceptibility to sepsis and the severity of the disease has been explored. The intracellular protein (NOD2) contributes to antibacterial effects through a variety of mechanisms, including cytokine responses, production of reactive oxygen species, upregulation of antimicrobial peptides, altered intracellular trafficking and enhanced killing of intracellular microbes.[212] Mutations of genes involved in the innate immune system (NOD2) have been reported to be associated with an increased sepsis rate in adults[164] and very low birth weight infants.[163]

Infection and resulting fever occurs in malignancy due to defects in the neutrophil phagocytic bactericidal activity functions. These defects become more pronounced with the chemotherapy induced neutropenia rendering such patients more susceptible to opportunistic pathogens.[213] Our hypothesis is that defects in macrophages function due to NOD2 mutation may become apparent during periods of neutropenia.

The number of febrile events was analysed by NOD2 genotype, and also presence and/or absence of neutropenia. The proportion of patients with febrile events did not differ by NOD2 status. Our findings suggest that overall susceptibility to infection resulting in fever is not affected by NOD2 genotype. It may be that longer periods of neutropenia are required to detect this defect.

Microbiologically documented infections with identified organisms accounted for only 10-20% of all episodes of fever.[214] Pneumonia and pulmonary infiltrates accounted for 15-30% of clinically and/or radiologically documented fevers.[215] The other 50% were considered as fever of unknown origin (FUO).[216] The prevalence of particular classes of microorganism positively detected in febrile patients was

analysed with respect to NOD2 status for the total number of febrile events, total episodes of neutropenia and total patients.

The analysis of febrile events in relation to positively detected organism (bacterial, viral and fungal), or the febrile events without microbiological identification was analysed. There was a statistical significant association of positive bacterial cultures with the mutant NOD2 genotype in the absence of neutropenia and a trend to an association with viral isolation regardless neutropenia. Culture negative febrile events showed statistically significant negative association in mutant NOD2 genotypes in the absence of neutropenia.

Functional studies revealed that the Arg702Trp (SNP8), Gly908Arg (SNP12) and L1007fsinsC (SNP13) are defective in their ability to respond to bacterial LPS and PGN. The frameshift L1007fsinsC variant showed a lack of responsiveness to LPS and PGN-induced NF-κB activation. While the Arg702Trp and Gly908Arg variants responded to LPS and PGN, but the response was significantly reduced because of diminished ability to activate NF-κB.[217]

NOD2 is expressed mainly in monocytes[131] and to lesser extent in other blood leukocytes[144] This along with the diminished LPS and PGN response in the SNPs 8 and 12 could explain our findings of increase prevalence to bacterial organisms in the mutant group in the presence of normal neutrophil count.

Fever due to unidentified organisms, occurred significantly less frequently in the NOD2 mutant group in our study. This could be explained by the organisms included in this category, e.g. possible fungal and the NOD2 association with bacterial infections as in most studies.[163, 164]

Bacterial sepsis incidence increased, while episodes of fever where no organism was cultures was proportionally   decreased in patients with mutant NOD2 genotypes when neutrophil count was within the normal range. The association with bacterial sepsis and NOD2 polymorphisms has previously been documented but change in incidence of FUO not commented upon. Since this finding was in the absence of neurtopenia our study may suggest that monocytes of patients with

mutated NOD2 have a defect in function as a result of the malignancy itself rather than only due to chemotherapy. This findings need to be explored in a larger study.

The frequency of the NOD2 mutation in our study was 8.46% for two SNPs only. It may be that this study would show more significant values if the three planned SNPs were identified. Other issues were the small number of documented fungal and viral infections in our study population.

Overall the Arg702Trp, Gly908Arg seems not to affect the course, duration and antimicrobial therapy of febrile events whatever the causative organism was.

In the absence of neutropenia there was a trend towards more HL removal in patients with NOD2 mutant genotype this could reflect the macrophage function. However other indications of outcome were not different in the NOD2 genotypes.

The occurrence of both incidences in the absence of neutropenia; significant positive bacterial cultures as a cause of a febrile event and the trend of HL removal in the mutant NOD2 genotype could suggest the neutrophil defect in antimicrobial function[218] and the altered response towards LPS/PGN.[219] Although we might have hypothesized this effect would be stronger in the presence of neutropenia, this was not the case. Haematological malignancy and chemotherapy may not have the same effect on neutrophils, monocytes or other leukocytes of different genotypes of NOD2. Nevertheless these finding confirms other studies associating NOD2 mutation to bacterial cultures.[164] Viral isolates sepsis in relation to NOD2 mutation need to be examined in a larger study.

4.8.   Conclusion

4.8.1.   In relation to sepsis our study suggests

4.8.1.1   An association between NOD2 (Arg702Trp, Gly908Arg) and:

Sepsis, in the NOD2 mutant genotype showed variable occurrence with different organisms either positive or negative isolates all in the presence of normal neutrophil count.

There was a significant increase incidence of sepsis with bacteria as the causative organism, while sepsis due to unidentified organisms was less common in patients with NOD2 mutation. There was a trend towards a greater likehood of hickman line removal in patients with NOD2 mutations

There was also a trend to more viral isolates from septic patients with NOD2 mutations and this incidence occurred regardless absence or presence of neutropenia.

4.8.1.2.   There was no association between NOD2 (Arg702Trp, Gly908Arg) and incidence or duration of neutropenic episodes between the NOD2 genotypes.

The incidence of febrile events per neutropenic episode showed no difference between NOD2 genotypes nor the prevalence of any particular isolates as cause of the febrile events per patient.

Our findings in relation to sepsis suggest that monocytes in patients with the mutant NOD2 become less responsive to bacterial components. Another suggestion could be that in this group immune response to bacterial invasion relay on monocytes alone, indicating monocytes function diminishes with malignancy in the presence of polymorphisms.

4.8.2.   In relation to haematological malignancy:

NOD2 mutation, in particular Arg702Trp have a significant association with the diagnosis of chronic leukaemia but this may have been due to the small number if patients in that group.

Chapter 5

NOD2 mutation and ALL outcome

5.1.   Introduction:

ALL is the most common malignancy in childhood,[51, 52] accounting for 30% of all cancers and 80% of all leukaemia. It comprises 2 lineages; B-cell and T-cell precursor. B-cell accounts for 75% of ALL, and is generally associated with better outcome than T-cell linage ALL which constitutes approximately 25%.[52] Complete remission (CR) can be achieved in 70-80% of patients but more than half are expected to relapse later, with only a minority sustaining a long lasting second remission. 10-20% die early during induction treatment, and around a further 10% are refractory to remission-induction programs.[55]

Whilst ALL differs genetically in children and adults, common mechanisms underlay the documented genetic alterations and the leukemic transformation of hematopoietic stem cells. Mechanisms include proto-oncogene expression and chromosomal translocations that generate fusion genes with oncogenic potential. These changes affect cell processes including the capacity for self-renewal, proliferation, differentiation and prevention of apoptosis by promoting resistance to death signals.[53]

Favourable chromosomal abnormalities such as high hyperdiploidy are more frequently found in children. While the translocation involving chromosome 9 (9;22), the Philadelphia chromosome which is associated with poor outcome, is found in 25% of adults compared to only 5% of children.[51]

Allogeneic stem cell transplantation (Allo-SCT) preferably from a HLA-matched related donor has shown superiority in prolonging initial CR in Ph(+) ALL, but not in

patients with other high-risk leukaemia (e.g. t(4;11)). Some studies indicate that hypodiploidy, near-diploidy or poor responders to initial remission-induction therapy may also benefit from transplantation, but the evidence is not definitive.[54, 59] Other work indicates that disease free survival and possibly overall survival (OS) is prolonged after transplantation in the high-risk ALL group who failed chemotherapy, but success is limited compared to AML.[220]

In previous studies the NOD2 gene mutation has been studied in relation to the outcome in Allo- SCT recipients in acute leukaemia patients with respect to, the impact of the mutation on graft versus host disease (GvHD), DFS, OS, and transplant related mortality (TRM). The relative risk varies depending whether either recipient, donor or both carry the NOD2/CARD15 mutation.[8, 9, 221]

Holler et al., reported an increased risk of GvHD; grade (III/IV) when the recipient carried the NOD2 mutation, a risk which further increases if only the donor is positive for the mutation. A dramatic rise in GvHD occurs when both recipient and donor carry the NOD2 mutation.[8] The cumulative incidence of 1-year TRM increased from 20% in wild type recipient/donor pairs to 49% if the recipient was NOD2 mutant, 59% with the donor gene mutation, and 83% when both donor and recipient are NOD2 mutant.[8] Holler et al confirmed this in a multicenter study performed two years later.[221]

Mayor et al., studied the risk of relapse and death after Allo-SCT in relation to the presence of the NOD2 mutation and the study showed no impact of NOD2 gene mutation on TRM and GvHD. However, the presence of NOD2 mutation in the recipient did reduce OS, and increased the incidence of disease relapse (significant in ALL) compared to wild type-type NOD2 group.[9]

5.2.   Study Rationale

NOD2 mutations result in its impaired function as an intercellular receptor to bacterial LPS and peptidoglycan (PGN) and the subsequent activation of the NF-

κB signaling cascade[93, 138, 139] leading to increased infections. In this study we aimed to investigate the effect of the NOD2 mutation on relapse rate and overall survival in a group of ALL patients who only received chemotherapy and no SCT to assess the impact of this mutation in the outcome of this cohort.

5.3.   Materials:

Samples were collected as a part of UKALL-12 trial –MRD (minimal residual disease) study, in adult patients (age 15-59 years) following a diagnosis of ALL. Diagnosis was based on clinical parameters, blood and/or bone marrow morphology as defined by World Health Organisation and the inclusion criteria for this trial (UKALL-12; Medical Research Council UK and Eastern Co-operative Oncology Group trial).

Data of patients (date of birth, sex, date for the commencement of treatment, immunophenotype and cytogenetics) were collected either from CTSU (Clinical trial service unit and epidemiological unit) Oxford or the local (referral) hospital or laboratory. Philadelphia status was determined by FISH analysis at the local cytogenetic laboratory where samples were collected and confirmed by molecular technique in the Royal Free Hospital (RFH) laboratory. The clinical outcome was obtained mostly through the clinician from referral centres.

The inclusion criteria of this study were; diagnosis of ALL B-cell precursor and Ph negative. B cell precursor was chosen as it was the commonest in ALL with less chances of transplant. Allogenic transplant was an exclusion criterion.

5.4.   Methods:

General Methods are described in chapter 2.

From stored DNA samples in the molecular biology laboratory at the RFH, 60 samples were analyzed by following methods: PCR amplification using NOD2-WT and SNP fluorescently labelled primers, and genescanning. A random sample were amplified and sequenced after being genescanned to ensure the accuracy of the results.

Analysis was performed using Microsoft office excel 2007, P value was calculated by Chi square contingency table analysis. The OS, DFS (relapses) and time to achieve complete remission (CR) were calculated by Kaplan Meier method (SPSS 17.0).

5.5.   Results:

5.5.1.   Demographics

Patient demographics are shown in table 3.1. Males accounted for 55.32% aged between 18-49yrs, while females account for 44.68% aged between 16-55yrs.

The median age in males was 33.5 years and in females was 35.5 years, (table 5.1).

Table 5.1.   Patients' demographics.

Total samples 47	**Male**	**Female**
Number of samples	26	21
Percentage	55.32%	44.68%
Age range	18-49	16-55
Median age	33.5	35.5

Table 5.2   Causes of exclusions in the 13 samples/patients.

Cause of exclusion	Number of patients
Allogeneic SCT	8
T cell ALL	3
Unknown Outcome	2

5.5.2. Frequency of NOD2/CARD15 mutation:

Out of the 60 samples only 47 met the criteria, (table 5.2.). In the 47 DNA samples included 31 (65.96%) showed no mutation in any of the SNPs tested and 16 (34.04%) were mutated for any of the NOD2 SNPs. One of the DNA samples exhibited compound heterozygosity (SNP8 and 12), whilst only one sample was homozygote for SNP13, (table 5.3.).

Frequency of mutation in relation to sex showed no statistical significance, 8 (30.77%) out of the 26 males and 8 (38.10%) out of the 21 females showed mutation, (table 5.4.).

Table 5.3. The frequency of NOD2-SNPs mutations in 47 ALL DNA samples, the only homozygote mutation was in SNP13 and on e sample exhibited compound heterozygosity.

Genotype	SNP8(%)	SNP12 (%)	SNP13 (%)
Wild type	42 (89.36%)	37 (78.72%)	45 (95.74%)
Heterozygote	5 (10.34%)	10 (21.28%)	1 (2.13%)
Homozygote	0	0	1 (2.13%)

Table 5.4. Mutation in different sexes. P value was calculated by Chi squre.

Total samples 47	Males = 26	Females = 21
Wild type NOD2 =31	18 (69.23%)	13 (61.90%)
Mutant NOD2 =16 (P value 0.598)	8 (30.77%)	8 (38.10%)

5.5.3. Follow-up period:

Follow up in all 47 samples ranged between 111-1840 days with a median of 666.91 days until death or the completion of the study (last follow up). The earliest relapse occurred at 117 days, while the earliest death was at 169 days, both events occurred in patients with the wild type NOD2.

5.5.4. Outcome of ALL patients:

The outcome was analaysed in three groups, as time to achieve complete remission (TtCR), relapse and overall survival (OS). Each of the outcomes was analysed in relation to gender and NOD2 status separately and then combined.

The outcome was first analysed according to gender, (table 5.5.). The incidences of relapse and OS along with TtCR showed no differences between both genders. P value showed no significances between sexes in all outcomes (CR, relapse, OS).

35 patients initially entered CR but three (two males and one female), did not sustain remission and later died. One male and one female death were due to sepsis.

Table 5.5. Outcome in relation to gender. P value was calculated by Chi square & Fisher exact.

Outcome	Male (%)	Female (%)
47 samples	26 (55.32%)	21 (44.68%)
35 CR (P value 0.668)	20 (76.92%)	15 (71.43%)
12 Relapse (P value o.668)	6 (23.07%)	6 (28.57%)
10 Deaths (OS) (P value 0.703)	5 (19.23%)	5 (23.81%)

Table 5.6. Outcome in relation to NOD2 mutation. P value was calculated by Chi square & Fisher exact.

Total samples 47	Wild type NOD2 n=31 (65.96%)	Mutant NOD2 n=16 (34.04%)
35 CR P value 0.952	23 (74.19.%)	12 (75%)
12 Relapse P value 0.952	8 (25.81%)	4 (25%)
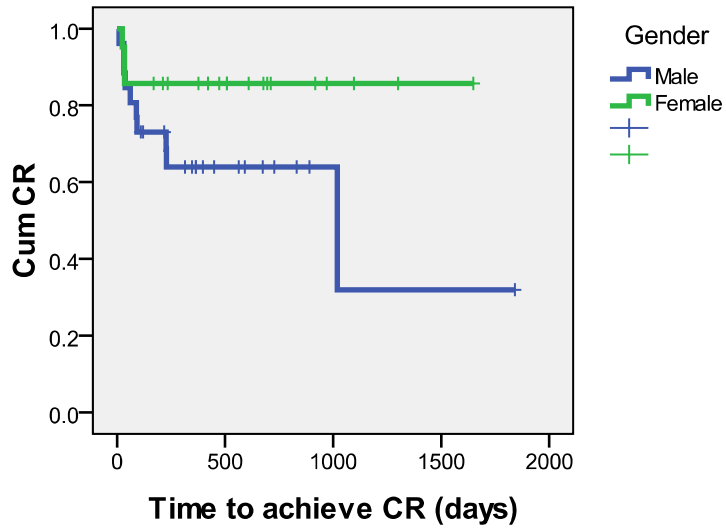
Figure 5.1.1.

Time to achieve complete remission in relation to gender. Male; 61.5% Female: 85.7 %(in proportion; proportion is a 100 of percentage).

P=0.077
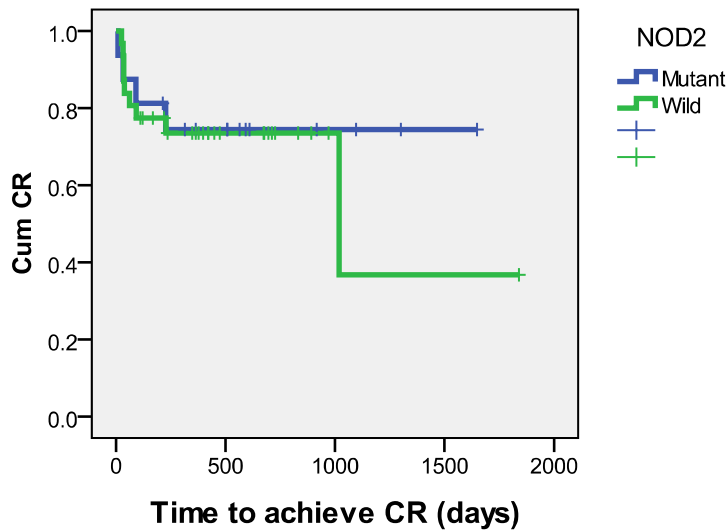
Figure 5.1.2.

Time to achieve complete remission in relation to NOD2 status.   Wild type:71% Mutant: 75%

(in proportion; proportion is a 100 of percentage).

P=0.679
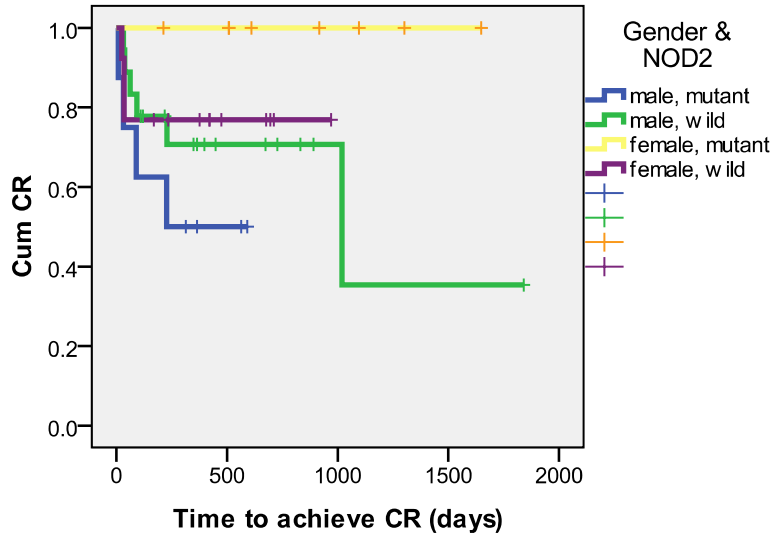
Figure 5.1.3.

Time to achieve complete remission in relation to gender and NOD2 status in combination.
Male wild type:66.7%, & mutant:50%.
Female wild type:76.9%, & mutant:100%
(in proportion; proportion is a 100 of percentage).
P=0.099

There were 35 CR, 74.19% and 75% in the wild type and mutant genotypes respectively, (table 5.6.). Only 32 sustained CR out of the total 35 (22 (70.97%) in the wild type NOD2 group and 10 (62.5%) in the mutant for any of the NOD2-SNPs. The TtCR did not show statistical differences in relation to either gender, NOD2 status or combined, (table 5.7., figures 5.1.1., 5.1.2., 5.1.3.).

There was no difference in the relapse rate between wild type and mutant NOD2 patients. Out of the 47 patients, 12 relapsed; 8 (25.81%) were mutated and 4 (25%) were wild type for NOD2, (table 5.6.). Statistically there was no significant differences in comparing incidence of relapse in relation to gender, NOD2 genotypes and combined, (table 5.8., figures 5.2.1., 5.2.2., 5.2.3.)

As noted above, three patients were initially in remission and subsequently died. Two of the patients initially in CR who later died were found to be mutant for the NOD2 gene, (table 5.9.). The cause of death in one was sepsis, and in the other transformation to AML. The third patient dying in the CR group was wild type for NOD-2 with unknown cause of death (table 5.10.).

There were 10 deaths in total, 3 were in CR while 7 were in relapse, (table 5.9.).
The cause of death in the wild type NOD2 patients were all as a result of the
relapsed disease, but in the mutant patients the causes of death varied, (table
5.10.). Overall survival at 2 years was 80.6% and 75% in the wild type and mutant
NOD2 respectively. Observation and follow up was stopped and deaths occurring
later had not been notified.

Overall survival did not show any statistical differences in all the categories, (table
5.11, figures 5.3.1., 5.3.2., 5.3.3.)

Table 5.7. Cox Regression analysis of the time to achieve complete remission (TtCR)

Analysis	P value	Odd ratio	CI 95%
Univariate Sex NOD2	0.09 0.68	0.33 1.28	0.09-1.21 0.39-4.22
Multivariate Sex NOD2	0.10 0.99	0.33 1.01	0.09-0.24 0.30-3.37

Table 5.8. Cox Regression analysis of the time to relapse.

Analysis	P value	Odd ratio	CI 95%
Univariate Sex NOD2	0.83 0.76	1.145 1.24	0.33-3.97 0.319-4.78
Multivariate Sex NOD2	0.79 0.76	1.18 1.27	0.323-4.97 0.336-4.15

Table 5.9. Deaths in both CR and relapse for wild type and mutant NOD2 patients. P value
were calculated by Fisher exact.

Deaths	Wild type NOD2; n=31	Mutant NOD2; n=16
Total deaths P value 0.654	6 (19.35%)	4 (25%)
Deaths in CR P value 0.218	1 (2.23%)	2 (12.5%)
Deaths in Relapse P value 0.741	5 (16.13%)	2 (12.5%)
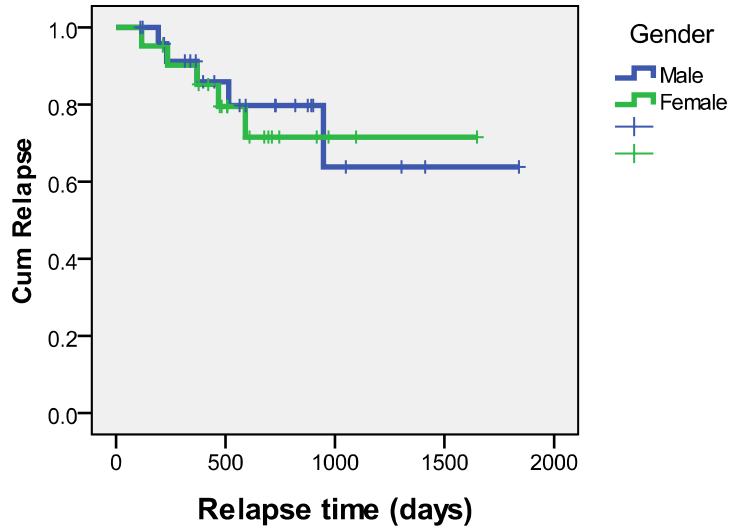
Figure 5.2.1.

Time to relapse in relation to gender. Male: 80.8% Female:76.2%

(in proportion; proportion is a 100 of percentage).

P=0.831
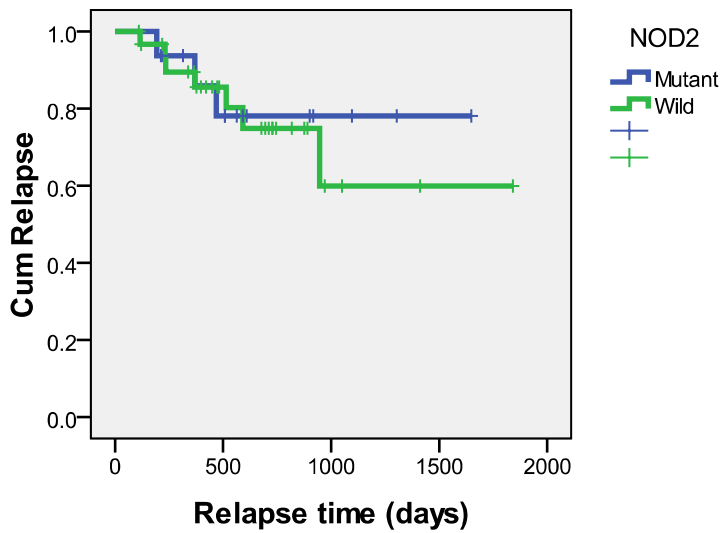
Figure 5.2.2.

Time to relapse in relation to NOD2 status.   Wild type:77.7% Mutnat:81.3%

(in proportion; proportion is a 100 of percentage).

P=0.758
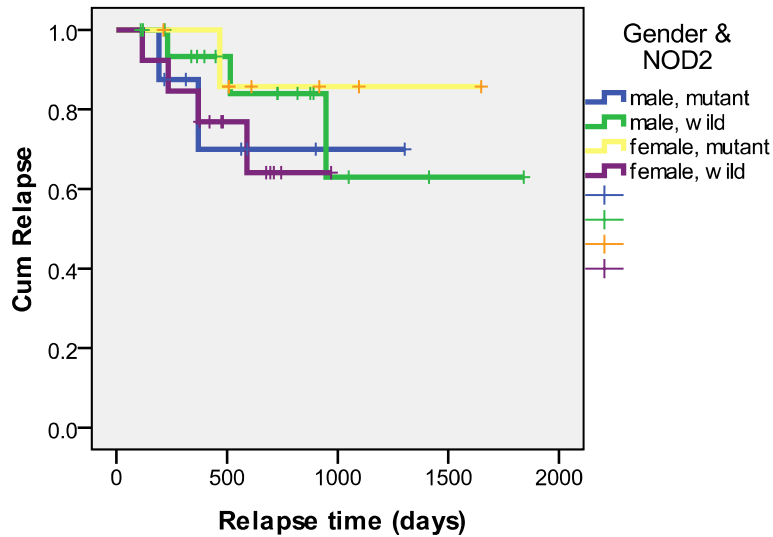
Figure 5.2.3.

Time relapse in relation to gender and NOD2 status in combination. Male wild type:83.3%, mutant:75%. Female wild type:69.2, mutant:87.5%

(in proportion; proportion is a 100 of percentage).

P=0.697

5.5.5. Survival of ALL patients by NOD2 genotype

Disease-free survival in the relapsed patients with a wild type NOD2 genotype ranged from 117-947 days, while for patients with the NOD-2 mutant genotype the range was between 193-917 days, (figure 5.2.2.). The odd ratio of disease-free survival in the relapse group was 1.24 and the 95% CI of ratio between o.319-4.78. NOD2 variants showed no propotional significance in the relapse group, p=0.76 (table 5.8., figure 5.2.2.).

The overall survival odd ratio 0.92 with 95% CI ranged between 0.259-3.289. Days of disease-free survival were in the range of 169-832 in the wild type genotype and 217-917 in the mutant genotype. P value 0.90 showed no significance. (table 5.9., figure 5.3.2.).

Neither of the compound heterozygote or the homozygote patients segregated with the death or relapse groups.

Complete remission achievement in relation to NOD2 status showed no significant differences p=0.68 with odd ratio of 1.28 and CI 95% 0.09-4.22. (table 5.7., figure 5.1.2.).

Table   5.10.   Cause of death in the four NOD2 mutant patients.

Patient-1	Patient-2	Patient-3	Patient-4
Female Relapse/sepsis	Male Autograft/CR/Sepsis	Male AML transformation	Female Post-autograft

Table 5.11.   Cox Regression analysis of the overall survival time (OS).

Analysis	P value	Odd ratio	CI 95%
Univariate Sex NOD2	0.70 0.90	1.27 0.92	0.366-4.436 0.259-3.289
Multivariate Sex NOD2	0.72 0.98	1.27 0.981	0.349-4.610 0.262-3.669
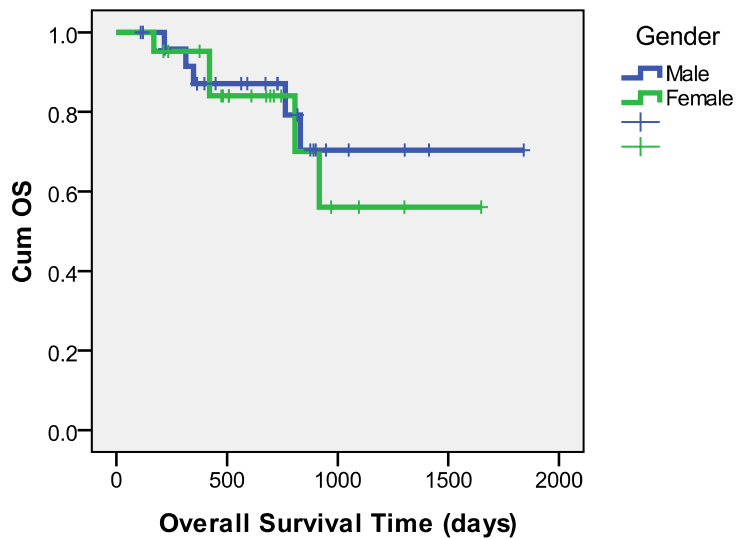
Figure 5.3.1.

Overall survival (OS) time in relation to gender. Male: 80% Female:76.2%

(in proportion; proportion is a 100 of percentage).

P=0.702

153
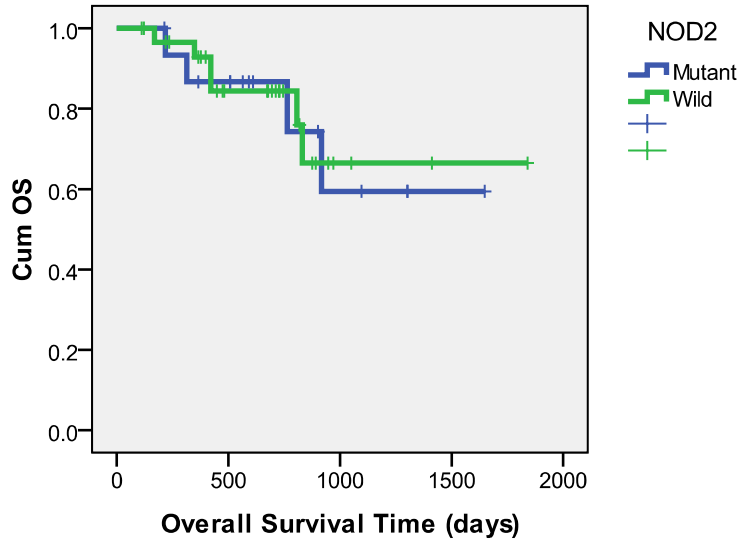
Figure 5.3.2. Overall survival (OS) time in relation to NOD2 status. Wild type:80.6% Mutant:75%

(in proportion; proportion is a 100 of percentage).

P=0.900
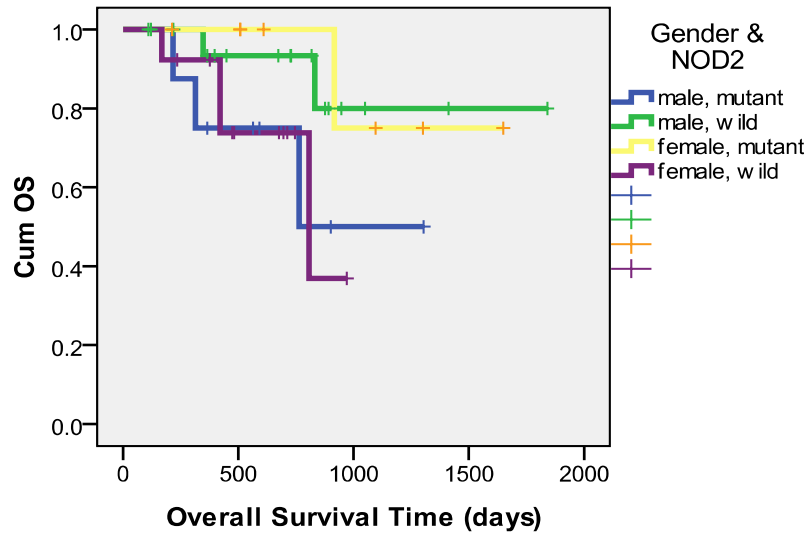
Figure 5.3.3. Overall survival (OS) time in relation to gender and NOD2 status in combination. Male wild type:88.9, mutant:62.5%. Female wild type:69.2%, mutant:87.5%

(in proportion; proportion is a 100 of percentage).

P=0.164

5.6.　　　Discussion:

Previous studies of mutations in NOD2 and their relation to relapse; disease free survival and overall survival have been performed in post-transplant patients for whom the indication for transplantation was leukaemia. In these studies the NOD2

status was investigated in recipients, donors or both to find if the mutation had a cumulative effect on the incidence of relapse, DFS ,OS, GvHD.[8, 9, 221] Other studies had linked NOD2 and different types of malignancies including colorectal,[204, 222] breast,[160, 161] NHL,[162] and MALT.[159]

The purpose of the current study was to examine the frequency of mutation in our ALL group and to investigate the impact of the NOD2 mutation on relapse rate and OS in a non-transplant group.

The incidence of the NOD2 mutation in this study was 34.04% for any of the NOD2 SNPs. This frequency of mutation is comparable to the study by Cuthbert et al. in which the frequency of the NOD2 mutation in a selected cohort in UK was 35.8%.[149] The NOD2 mutation were slightly higher in the females than males with percentage of 38.10 and 30.77, respectively; but occurrence differences maybe need to be validated on a larger cohort of patients.

In our study the ALL incidence was slightly higher in males (55.32%) and in younger age than in female (mean age of 34.85 in males). Recent studies have shown that, males show an increased occurrence of ALL.[52, 59] Males ≥ 30years have a worse outcome[61] but in our study relapse incidence and OS were equal in both sexes 50% for all groups (wild type and mutant genotype). CR was between 42.86-57.14% (female-male) with a mean follow up period of 22.02 months and this could be comparable to the 5 years survival of 20-40% observed by Castagnola et al.[55]

We observed no significant difference in CR, incidence of relapse or OS among all groups. Achievement of complete remission and relapse showed no differences between NOD2 variants. Death occurred less frequently in the mutant (40%) than the wild type group (60%). This contrasts with other studies which showed an increase in incidence of relapse and a decrease in overall survival in the mutant group[8, 9, 221] but is in agreement with other investigators.[10]

In the group of patients who did not survive 2 (NOD2 mutant) out of the ten (20%) deaths were due to sepsis which may confirm the immunological effect theory of

this gene and which we are going to investigate it further in our study in different population.

5.7. Conclusion

We detect no impact of NOD2/CARD15 mutation on OS and relapse in non-transplanted Philadelphia negative -B cell ALL and we found no association between the presence of mutation and sex.

Chapter 6

Genetic determinant of sepsis in haematological malignancy

Combined study:

NOD2 & Chitotriosidase mutations

6.1.   Introduction

Host defence against invading microorganisms is elicited by immune cells.[93, 94] The phagocytic and antigen presenting cells such as neutrophils and macrophages express various receptors for detection of such microorganisms. The extracellular toll-like receptors (TLR) recognise pathogen associated molecular patterns (PAMPs)[94] and intracellular NOD2 receptors bacterial derived LPS and PGN.[93, 131, 144] Chitotriosidase synthesis by macrophages or neutrophil granules occurs in response to stimuli by chitin containing fungi, however its role in fungal defence is not completely clear.[98, 99]

Mutations in the innate immunity genes (chitotriosidase and NOD2) have been under focus. Studies show an impaired immune response and resultant defective NF-κB activation initiates the inflammatory response in Crohn's disease patients carrying the NOD2 mutation.[4, 131] Mutation in NOD2 also was found to reduce phagocytosis and increases susceptibility to bacterial infections[163, 175] as well to increase mortality in septic patients.[163, 164] On the other hand chitotriosidase levels have been seen to rise in bacterial, fungal[6, 7] and malaria falciparum infections.[112] Chitotriosidase mutations appear to render patients susceptible to gram negative infections[96] and *W. bacrofti*.[115]

Recently a study was conducted to elicit the effect of TLR and NOD2 on chitotriosidase expression and release as part of the innate immune response. Results showed NOD2 activation induces chitotriosidase expression on the macrophages while TLR regulates its release from neutrophils in response to bacterial MDP.[97]

In contrast in this study we have attempted to uncover the synergistic effects of both gene mutations in the innate immune response required for elimination of infections (bacterial, fungal or viral). The study was conducted in the same population as previous studies. Patients with a diagnosis of haematological malignancy in relation to the absolute neutrophil count (neutropenic or non-neutropenic), were observed during their hospital stay. Events of fever (as defined previously) were investigated and treated.

Gene mutations (chitotriosidase alone, NOD2 alone or mutation of both genes in an individual) in relationship to infection, sepsis and absolute neutrophil count were studied in this group of patients. The infectious pathogen and outcome of infection were analysed according to mutant status.

6.2.   Hypothesis:

Patients carrying combined mutations of both chitotriosidase and NOD2 are at increased risk of infection and sepsis during periods when they are neutropenic.

6.3.   Objectives:

1) To determine the effect of mutations (chitotriosidase, NOD2 or in combination) on the incidence and outcome of sepsis in patients with haematological malignancy, including those with leukaemia, lymphoma, and myeloma.

2) To examine the effect of mutations (chitotriosidase, NOD2 or in combination) on the incidence and outcome of sepsis in patients with haematological malignancy who are neutropenic and those who are not.

3) To examine the effect of mutations (chitotriosidase, NOD2 or in combination) on the prevalence of organisms in neutropenic and non-neutropenic patients with haematological malignancies.

4) To correlate neutrophil function and duration of neutropenia with the mutations (chitotriosidase, NOD2 or in combination).

6.4. Materials and methods:

General material and methods are described in Chapter 2.

Inclusion and exclusion criteria are also described in chapter 2.

Recruitment of patients with haematological malignancy was performed in the Royal Free Hospital.

The study was ethically approved and all patients gave informed consent. They were followed up during their in-patient stay. Clinical data were collected daily by the clinical fellow. Observations were recorded including ANC, temperature, related investigations (microbial and imaging) to determine the source and type of sepsis, and antimicrobial management.

Investigation and data of patients recruited from the out-patient department or after patients discharge were recorded electronically or from paper medical records.

DNA was extracted and genotyped for chitotriosidase and NOD2, according to methods mentioned in chapter 2. Only two SNPs (8 and 12) were studied in relation to NOD2 mutation.

6.5.   Statistics:

The p value was calculated by the Chi square and Kruskal Wallis tests, a p value of ≤0.05 considered significant.

(http://www.physics.csbsju.edu/stats/contingency_NROW_NCOLUMN_form.html)

The p value differences were calculated for four proportions. The wild type for any mutation, versus chitotriosidase only mutation, versus NOD2 only mutation, versus combined gene mutation.

Basic descriptive statistics and graphs were prepared using Microsoft excel and SSPS 17.0.

6.6.   Results:

6.6.1.   Incidence of combined gene mutation:

Mutation: Out of the 201 patients, 7 (3.48%) were mutant for both chitotriosidase and NOD2. The proportion of a solitary mutation of either gene was 37.31% for chitotriosidase and 4.97% for NOD2 (table 6.1.).

6.6.2.   Range of Diagnoses

The study population was 201 patients. The range of diagnosis varied. There was no difference in diagnosis by mutation (table 6.1.).

6.6.3.   Analysis of episodes of neutropenia (EoN):

Patients with a diagnosis of haematological malignancy and receiving chemotherapy become neutropenic. A fall of the absolute neutrophil count (ANC) ≤ 0.5×10^9/L is referred to as neutropenia. Neutropenia was analysed according to its

incidence and duration. The number of patients developing neutropenia and the frequency of neutropenic episodes per patient was also analysed. Analysis was done in four proportions in the wild type for any mutation, chitotriosidase only mutation, NOD2 only mutation and the combination of mutation for chitotriosidase and NOD2.

Table 6.1.   Diagnosis with different haematological malignancies in our study population. The number of patients and frequencies (%) in: total, in wild type to any, in relation to chitotriosidase only mutation, NOD2 only mutation or both mutations. P value calculated by Chi square.

Mutation status	Diagnosis				
	Lymphoma	Multiple myeloma	Acute Leukaemia	Chronic Leukaemia	Other
Total 201; (%)	106 (52.74%)	32 (15.92%)	50 (24.88%)	11 (5.47%)	2 (0.99%)
WT for any mutation: 109 (54.23%)	59 (54.13%)	17 (15.60%)	25 (22.94%)	6 (5.50%)	2 (1.83%)
Chitotriosidase: 75 (37.31%)	38 (50.67%)	13 (17.33%)	22 (29.33%)	2 (2.67%)	0
NOD2: 10 (4.97%)	7 (70%)	0	1 (10%)	2 (20%)	0
Both: 7 (3.48%)	2 (28.57%)	2 (28.57%)	2 (28.57%)	1 (14.29%)	0
P=	0.384	0.415	0.522	0.099	0.636

6.6.3.1.   <u>Number of patients with neutropenic episode per genotype:</u>

The total proportion of patients developed episodes of neutropenia (wild type for any mutation chitotriosidase and NOD2) was 93 (85.32%) out of 109. For chitotriosidase mutant group 60 out of 75 (80%) and the NOD2 mutant group 7 out of the 10 patients (70%) became neutropenic during their treatment. 5 out of 7 (71.43%) of the individuals carrying both mutations became neutropenic. There were no statistically significant differences between the four proportions (table 6.2.).

6.6.3.2.   <u>Number of neutropenic episodes per genotype:</u>

There were 450 episodes of neutropenia in total, 261 episodes out of the 450 occurred in patients wild type for any of the mutations, in the 158 chitotriosidase

only mutation genotypes and 18 in the NOD2 only mutation patients. 13 out of the 450 episodes occurred in the patients group carrying both mutations (table 6.3.)

Table 6.2. Total number of patients (Pts) with episodes of neutropenia (EoN) in each group (wild type for any mutation, chitotriosidase only mutation, NOD2 only mutation and combined genes mutation). % of neutropenic patients within each genotype. P value calculation by Chi square.

Total patients in the study =201	WT for any of the mutations	Chitotriosidase only mutation	NOD2 only mutation	Combined mutations
Total patients (%)	109 (54.23%)	75 (37.31%)	10 (4.97)	7 (3.48%)
Pts with EoN in each genotype (P= 0.470)	93 (85.32%)	60 (80%)	7 (70%)	5 (71.43%)

Table 6.3. Episodes of neutropenia (EoN) analysed for wild type for any mutation, chitotriosidase only mutation, NOD2 only mutation and combined mutation proportions.

Total episodes of neutropenia =450	WT for any of the mutations EoN=261	Chitotriosidase only mutation EoN=158	NOD2 only mutation EoN=18	Combined mutations EoN=13
Total pts / genotype	109	75	10	7
Total neutropenic episodes	261	158	18	13
Total neutropenic days	2496	1510	174	88
Range Episodes of neutropenia / patient	0-11	0-13	0-4	0-6
Neutropenic days per patient	0-80	0-50	0-33	0-23
Median Episodes of neutropenia/patient	2	2	1.5	2
Neutropenic days/patient	11.5	10	14.5	9
Day/episode	5.75	5	9.67	4.5

6.6.3.3.   Duration of neutropenic episode:

The duration of neutropenic episodes in the four groups was calculated, in order to evaluate the effect of single or combined mutation on neutrophil regeneration.

The total number of neutropenic days were 2496, 1510, 174 and 88 in the wild type for any mutation, chitotriosidase only mutation, NOD2 only mutation and combined gene mutations genotypes respectively (table 6.3.).

The median number of neutropenic days per patient (14.4) and neutropenic days per episode (9.67) were higher in the NOD2 only mutation group. Again, although suggestive, there were no statistically significant differences between the groups (table 6.3.).

Overall we found no statistically significant differences between the different groups in the occurrence of neutropenic episodes per genotype (table 6.2.).

6.6.4. Analysis of febrile events:

Analysis was performed to determine if the type of mutation affect the frequency of fevers. We analysed the incidence of febrile events in the four groups (wild type for any, chitotriosidase alone, NOD2 alone or in combination) in relation to presence and/or absence of neutropenia.

6.6.4.1. Febrile neutropenic events (FNE):

There was a total of 307 febrile events. 195 (63.52%) febrile events occurred in the presence of neutopenia while 112 (36.48%) occurred in the absence of neutropenia (table 6.5., 6.6.).

There were no statistical differences in the proportion of patients developing febrile events with neutropenia only, without neutropenia, either neutropenic or non-neutropenic events or both events (neutropenic and non-neutropenic events) for all genotype groups (table 6.4.).

The number of febrile neutropenic events per patient, per episode of neutropenia and per days of neutropenia was almost the same in the NOD2 and combined mutation groups. There were no statistical differences in the occurrence of febrile neutropenic events in the three groups (table 6.5.).

Table 6.4. Porportions of patients (Pts) out of each group with febrile events whilst neutropenic (FNE), non-neutropenic (FNnE), either (FNE or FNnE) or both (FNE and FNnE) febrile events. The p value calculated by the Chi square test compares the wild type for any mutation: chitotriosidase only mutation: NOD2 only mutation: combined mutation proportions.

Total	WT for any of the mutations	Chitotriosidase only mutation	NOD2 only mutation	Combined mutations
Total pt 201 (%)	109 (54.23%)	75 (37.31%)	10 (4.98%)	7 (3.48%)
Pts with FNE (% pts of FNE/ genotype). P = 0.492	64 (58.71%)	37 (49.33%)	6 (60%)	5 (71.43%)
Pts with FNnE (% pts of FNnE/ genotype). P = 0.860	43 (39.45%)	26 (34.67%)	3 (30%)	3 (42.86%)
Pts with either febrile events P = 0.815	49 (44.95%)	35 (46.67%)	5 (50%)	2 (28.57%)
Pts with both febrile events. P = 0.346	30 (27.52%)	14 (18.67%)	2 (20%)	3 (42.86%)

Table 6.5. Number of febrile neutopenic events (FNE) in the wild type for any, chitotriosidase only mutation, NOD2 only mutation and combined mutation proportions.

Total febrile neutropenic events = 195	WT for any of the mutations	Chitotriosidase only mutation	NOD2 only mutation	Combined mutations
Number of FNE	112	66	7	10
Range per patient	0-5	0-7	0-2	0-4
Median Number of events/patient Event/episode of neutropenia Event/neutropenic days	1 0.5 0.087	0 0 0	1 0.67 0.069	1 0.5 0.11

Table 6.6. Number of febrile non-neutropenic events (FNnE) analysed in the wild type for any, chitotriosidase only mutation, NOD2 only mutation and combined mutation proportions.

Total febrile non-neutropenic events=112	WT for any of the mutations	Chitotriosidase only mutation	NOD2 only mutation	Combined mutations
Number of FNnE	66	36	5	5
Range per pt	0-6	0-4	0-2	0-2
Median; number of events per pt	0	0	0	0

6.6.4.2. Febrile non-neutropenic events (FNnE):

112 events of non-neutropenic fever occurred. The number of patients with febrile events not associated with neutropenia were 43 out of the 109 patients wild type

for any mutation (39.45%), 26 out of the 75 in the chitotriosidase mutant genotype (34.67%), 3 out of the 10 in the NOD2 mutant genotype (30%) and 3 out of 7 in patients with both gene mutation (table 6.4. & 6.6.).

6.6.4.3.   Analysis of patients developing either febrile (neutropenic or non-neutropenic) event:

There was no statistical significance in the proportion of patients who developed any febrile event either in the presence or absence of neutropenia.

There were 49 (44.95%) in the wild type for any mutation, 35 (46.67%) patients in the chitotriosidase only mutation, 5 (50%) in the NOD2 only mutation and 2 (28.57%) in the combined gene mutation group (table 6.4.).

6.6.4.4.   Analysis of patients developing both febrile (neutropenic and non-neutropenic) events:

The number of patients developing febrile events both while they are neutropenic and non-neutropenic during their course of therapy was 30 (27.52%) in the wild type for any mutation, 14 (18.67%) in the chitotriosidase only mutant, 2 (20 %) in the NOD2 only mutant and 3 (42.86%) in the combined mutation genotypes (chitotriosidase & NOD2 mutant). There were no statistical differences between the four proportions (table 6.4.).

6.6.5.   Analysis of the types of organism cultured

The likelihood of having positive cultures with a particular organism (bacteria, virus or fungus) was analysed according to the number of febrile events, the number of episodes of neutropenia and the number of patients within the four proportions.

Table 6.7.1. Total febrile events regardless neutropenia in the four groups and the type of identifiable organism if present. P value by Chi square test.

Total febrile events = 307	WT for any of the mutations =178	Chitotriosidase only mutation =102	NOD2 only mutation =12	Combined mutations =15
Total bacterial +ve events Bacterial +ve/febrile event % P= 0.123	86 48.31%	61 59.80%	9 75%	8 53.33%
Total fungal +ve events Fungal+ve/febrile event % P =0.298	5 2.81%	0	0	0
Total viral +ve events Viral +ve/febrile event % P = 0.116	1 0.56%	2 1.96%	1 8.33%	0
Total culture -ve events Culture –ve / febrile event % P = 0.094	86 48.31%	39 38.24%	2 16.67%	7 46.67%

Table 6.7.2 Total febrile neutropenic events in the four groups and the type of identifiable organism if present. P value by Chi square test.

Total febrile neutropenic events (FNE) =195	WT for any of the mutations FNE=112	Chitotriosidase only mutation FNE=66	NOD2 only mutation FNE=7	Combined mutation FNE=10
Number of bacterial FNE Bacterial rate/FNE, % P =0.485	63 56.25%	41 62.12%	5 71.43%	4 40%
Number of fungal FNE Fungal rate / FNE,% P= 0.683	2 1.79%	0	0	0
Number of viral FNE Viral +ve / FNE, % P = 0.016	0	2 3.03%	1 14.29%	0
Number of culture -ve FNE Culture -ve rate / FNE, % P = 0.212	47 41.96%	23 34.85%	1 14.29%	6 60%

6.6.5.1. Analysis of organism cultured per febrile events in the presence and/or absence of neutropenia.

Here the number of febrile events in relation to the organism isolated was calculated in order to evaluate organism prevalence, in the four different genotypes in the presence and/or absence of neutropenia.

Analysis of the number of febrile events with identified organism (bacterial, fungal or viral) showed no statistical differences regardless neutropenia in all four genotypes. Analysis of the number of febrile events and unidentified organism showed a trend towards significance with the highest (48.31%) occurrence in the wild type for any proportion and lowest (16.67%) in the NOD2 only mutation (table 6.7.1.).

Analysis of febrile events in the presence of neutropenia in relation to organism showed a statistical significance when viruses were the isolate. The diagnosis of positive viral isolate was higher in the NOD2 only mutation and none in the combined gene mutation and wild type for any genotypes, however the clinical significance in unclear as only a very small number of viral isolates were identified (table 6.7.2.).

Analysis of febrile events in the absence of neutropenia showed statistical significance with positive bacterial cultures, lowest in the wild type group. Cultures negative fever for were highest in the wild type group and lowest in patients with mutations.

Table 6.7.3.   Total febrile non-neutropenic events in the four groups and the type of identifiable organism if present. P value by Chi square test.

Total febrile non-neutropenic events (FNnE) =112	WT for any of the mutations FNnE=66	Chitotriosidase only mutation FNnE=36	NOD2 only mutation FNnE=5	Combined mutations FNnE=5
Number of bacterial FNnE Bacterial rate/FNnE% P = 0.017	22 33.33%	20 55.56%	4 80%	4 80%
Number of fungal FNnE Fungal rate / FNnE% P =0.542	3 4.54%	0	0	0
Number of viral FNnE Viral +ve / FNnE% P =0.872	1 1.52%	0	0	0
Number of culture negative FNnE Culture -ve rate / FNnE% P = 0.076	40 60.61%%	16 44.44%	1 20%	1 20%

6.6.5.2. Analysis of organism cultured per episode of neutropenia

Patients who develop neutropenia are prone to develop infections and become febrile. However not all patients develop fever whilst neutropenic. The reason for this is likely to be multifactorial with involvement of innate and adaptive immunity compensating for neutropenia. In this section the proportion of episodes of neutropenia during which patients became febrile for each genotype was calculated.

There was statistical difference in the number of neutropenic episodes with fever due to unidentified organism (FUO) in the four groups. Neutropenic episodes which were culture negative were higher when both genes were mutant in combination (table 6.8.). Episodes of neutropenia and viral isolate were evident with either mutation.

There was no significant difference between the total episodes of neutropenia due to bacteria or proven and probable IFD in the four groups (table 6.8.).

Table 6.8. Total episodes of neutropenia associated with febrile events in the four groups and the type of identifiable organism if present. P value by Chi square test.

Episodes of neutropenia with febrile events.	WT for any of the mutations; Total EoN =261	Chitotriosidase only mutation; Total EoN =158	NOD2 only mutation; Total EoN =18	Combined mutations; Total EoN =13
EoN with bacterial event. FNE (%). P = 0.696	56 (21.46%)	37 (23.42%)	6 (33.33%)	3 (23.08%)
EoN with probable/ proven fungal event. FNE. (%). P =0.693	2 (0.77%)	0	0	0
EoN with viral event. FNE (%). P = 0.027	0	2 (1.27%)	1 (5.56%)	0
EoN with culture negative event. FNE. (%). P = 0.017	42 (16.09%)	24 (15.19%)	1 (5.56%)	6 (46.15%)

6.6.5.3. Analysis of organism cultured per patient

In this section all patients developing febrile events were categorised according to the organism grown in their cultures. This analysis was carried out in the presence and/or absence of neutropenia.

There were no statistically significant differences in the proportions of patients have positive cultures to bacteria or fungal isolates either when neutropenic or not neutropenic (table 6.9.1 & 6.9.2).

There was a trend towards significance in the prevalence of viral isolates with the presence of neutropenia in the NOD2 mutant patients but numbers were low. Another trend towards significance occurred in patients with febrile events regardless neutropenia (FNE, FNnE or both) and the diagnosis of FUO due to negative culture and isolates. This finding was higher in the wild type for any mutation group (table 6.9.3 & 6.9.4).

Patients with combined mutation seemed not to exhibit any organism more than the solitary mutation.

Table 6.9.1. Patients with febrile events and positive bacterial cultures. FNEs only, FNnEs only, any event either FNE or FNnE, and both FNE & FNnE. in the four groups. P value by Chi square test.

Fever and positive bacterial cultures	WT for any of the mutations; Total pts=109	Chitotriosidase only mutation; Total pts=75	NOD2 only mutation; Total pts=10	Combined mutations; Total pts=7
Patients with only FNE. (%). P = 0.871	30 (27.52%)	19 (25.33%)	3 (30%)	1 (14.29%)
Patients with only FNnE. (%). P =0.393	11 (10.09%)	8 (10.67%)	2 (20%)	2 (28.57%)
Patients with any events, either FNE or FNnE. (%). P =0.755	49 (44.95%)	34 (45.33%)	6 (60%)	4 (57.14%)
Patients with events, both whilst neutropenic & non-neutropenic. (%). P = 0.899	8 (7.34%)	7 (9.33%)	1 (10%)	1 (14.29%)

Table 6.9.2.   Patients with febrile events and proven or probable IFD. FNEs only, FNnEs only, any event either FNE or FNnE, and both FNE & FNnE. in the four groups. P value by Chi square test.

Fever with proven and probable IFD	WT for any of the mutations; Total pts=109	Chitotriosidase only mutation; Total pts=75	NOD2 only mutation; Total pts=10	Combined mutations; Total pts=7
Pts with only FNE. (%) . P= 0.636	2 (1.83%)	0	0	0
Pts with only FNnE. (%). P= 0.463	3 (2.75%)	0	0	0
Pts with total events either FNE or FNnE. (%). P =0.228	5 (4.59%)	0	0	0
Pts with events, both whilst neutropenia and non-neutropenia. (%). P =0.838	1 (0.92%)	0	0	0

Table 6.9.3.   Patients with febrile events due to viral pathogens. FNEs only, FNnEs only, any event either FNE or FNnE, and both FNE & FNnE. in the four groups. P value by Chi square test.

Fever and viral positive event	WT for any of the mutations; Total pts=109	Chitotriosidase only mutation; Total pts=75	NOD2 only mutation; Total pts=10	Combined mutations; Total pts=7
Pts with only FNE. (%). P= 0.061	0	2 (2.67%)	1 (10%)	0
Pts with only FNnE. (%) P= 0.838	1 (0.92%)	0	0	0
Pts with total events either FNE or FNnE. (%). P = 0.236	1 (0.92%)	2 (2.67%)	1 (10%)	0
Pts with fever, both whilst neutropenia and non-neutropenia. (%) P =0.636	2 (1.83%)	0	0	0

6.6.6. Course and outcome of the febrile events

The outcome of febrile events in the different groups was analysed, in the presence and absence of neutropenia.

There were 112, 66, 7 and 10 febrile neutropenic events in the wild type for any mutation, chitotriosidase only mutation, NOD2 only mutation and combine mutation groups respectively. Total febrile days (neutropenic or non-neutropenic) and total days of antimicrobial treatment per group showed no statistical differences between the four groups. Number of febrile events ended in recovery, ITU admission or death showed no significance between all groups (table 6.10.).

Combined gene mutation showed no effect in the duration of febrile events or treatment required.

The outcomes of such febrile events in the presence or absence of neutropenia showed no significant differences. Although a trend towards significance was observed in the febrile non-neutropenic patients with combined mutation and the necessity of Hickman line removal as a part of management.

Table 6.9.4. Patients with febrile events without identifiable organism. FNEs only, FNnEs only, any event either FNE or FNnE, and both FNE & FNnE. in the four groups. P value by Chi square test.

Fever and culture -ve event	WT for any of the mutations; Total pts=109	Chitotriosidase only mutation; Total pts=75	NOD2 only mutation; Total pts=10	Combined mutations; Total pts=7
Pts with only neutropenic events. (%) P= 0.578	24 (22.02%)	12 (16%)	1 (10%)	2 (28.57%)
Pts with only non-neutropenic events. (%). P= 0.550	19 (17.43%)	10 (13.33%)	1 (10%)	0
Pts with total events either neutropenia or non-neutropenia. (%). P= 0.055	54 (49.54%)	24 (32%)	2 (10%)	3 (42.86%)
Pts with fever, both whilst neutropenia and non-neutropenia. (%). P= 0.164	11 (10.09%)	2 (2.67%)	0	1 (14.29%)

Table 6.10. Outcome of febrile events in the four proportions. (FNE: febrile neutropenic event, FNnE: febrile non-neutropenic event, R: treatment, HL: Hickman line, pts: patients, ITU: intensive treatment unit). P values calculated by Chi square and Kruskal Wallis (Kw) tests.

Course & Outcome	FNE= 112:66:7:10				FNnE= 66:36:5:5			
Pts per group	WT; 109	Chito; 75	NOD2; 10	Comb; 7	WT; 109	Chito; 75	NOD2; 10	Comb; 7
Total febrile days/genotype P=	460 (Kw) 2.988	226	49	53	221 0.085	147	22	12
Total days of antimicrobial R P=	1477 Kw 1.830	792	136	121	733 0.111	424	65	39
Number of FE necessitated HL removal. P=	10 8.93% 0.959	7 10.61%	1 16.67%	1 10%	4 6.06% 0.078	5 13.89%	1 20%	2 40%
Number of FE with complete recovery. P =	105 93.75% 0.625	64 96.97%	7 100%	9 90%	60 90.91% 0.353	29 80.56%	5 100%	4 80%
Number of pts with recovered febrile events. P=	60 55.05% 0.507	35 46.67%	5 50%	5 71.43%	38 34.86% 0.797	21 28%	3 30%	2 28.57%
Number of febrile events ended in ITU. P=	5 4.46% 0.274	1 1.51%	1 14.29%	1 10%	4 6.06% 0.391	5 13.89%	0	1 20%
Number of pts admitted to ITU P=	5 4.59% 0.224	1 1.33%	1 10%	1 14.29%	3 2.75% 0.847	3 4%	0	0
Number of FE ended in death. P=	2 1.79 0.871	2 3.03%	0	0	2 1.79% 0.305	4 11.11%	0	0
Number of pts died. P=	2 1.83% 0.871	2 2.67%	0	0	2 1.83% 0.305	4 5.33%	0	0
Median -Fever days per patient -Antimicrobial R days per patient -Antimicrobial R days/fever days	2 9 4.5	0 0 0	1 7.5 7.5	7 15 2.14	0 0 0	0 0 0	0 0 0	0 0 0

6.7.　　　Discussion

In this study, we investigated the association between functional polymorphisms in selected genes involved in innate immunity and the risk of infections. The NOD2, a general sensor of PG from negative and gram positive bacteria[223] and chitotriosidase with the chitolytic activity towards fungal pathogens.[224]

The incidence of double mutation of both genes (chitotriosidase & NOD2) in our study population was 3.48%. This section of the study was done to determine the effect of combined gene mutation in innate immunity (chitotriosidase & NOD2) compared to single gene mutation or no mutation on the incidence and outcome of sepsis in the presence or absence of neutropenia compared to single mutation of each gene.

The presence of combined mutations (chitotriosidase & NOD2) showed no statistical differences in the incidence and duration of neutropenia as well as the number of neutropenic days per patient. Neutropenic days per patient and per episode of neutropenia were higher in the NOD2 mutation compared to the other three groups.

The number of febrile events was analysed per group, and in the presence and/or absence of neutropenia. The proportion of patients with febrile events did not differ statistically between the four groups. Also there were no statistical differences in the number of febrile events per episode of neutropenia. This section showed no impact differences between the four groups whether one gene or both gene were mutant on the number of febrile events per patient in the presence or absence of neutropenia or the in number of febrile events per episode of neutropenia.

The prevalence of certain pathogens were analysed in relation to number of events, patients and organisms.

Unidentified organisms causing fever in relation to episode of neutropenia was significantly higher in the combined gene mutation group.

Wild type for any mutation group seemed to have a tendency for unidentified cause of febrile events regardless or without neutropenia. In all comparisons NOD2 only mutation group was repeatedly the lowest in FUO occurrence.

Episodes of neutropenia and fever due to viral isolate were significantly higher in the NOD2 only mutation. This significance was carried along when febrile events associated with neutropenia were analysed.

It is worth mentioning that documented viral infections were very few in our study. Viruses are still difficult to isolate technically. The availability of viral RNA-PCR is still limited and not all viruses could be identified. For these reasons it could be that viral infection, reactivation or co-infection were missed and the diagnosis of FUO is eminent. As it is shown from our study usually patients are neutropenic and may be unable to elicit any immune response. Patients could deteriorate so fast prior to a diagnosis or occurrence of fever.

Fever due to viral isolates, especially in the presence of neutropenia and in relation to NOD2 mutation may need to be investigated in larger scale in the future.

In the absence of neutropenia both the combined gene mutant and NOD2 only mutant groups showed statistical significance of positive bacterial cultures as a cause of sepsis.

Fungal infections were so small in number to give a good picture in our study.

Combined genes mutation seems to have no effect in overall course and outcome of febrile events in comparison to only gene mutation. Although a trend of Hickman line removal was observed in the combined gene mutant group this could be due to increase defect in macrophages function.

In our study population the incidence of combined gene mutation was only 3.48%.

6.8   Conclusions

In this comparative study the following were significant associations:

Positive bacterial cultures and fever with normal count of neutrophils were higher in patients with NOD2 only mutation or combined gene mutations.

Positive viral isolate in fever accompanied with neuropenia in the group of NOD2 only mutation.

Culture negative and fever with neutropenia in the combined gene mutation group.

Chapter 7

General discussion

7.1. Genetic determinants of sepsis in haematological malignancy:

Studies are ongoing to determine the role of genetic background in various malignancies, infections, immunity and inflammatory syndromes, since some genes have been shown to be linked to incidence, course and outcome of such conditions either directly or indirectly.

Patients diagnosed with haematological malignancy are at risk of sepsis due to bone marrow involvement and the subsequent neutropenia. Studying the impact of genetic determinants may improve our understanding of the pathogenesis and ultimately may allow identification of patients who would benefit from specific preventive or therapeutic measures.

In our study we focused on two genes, which have been implicated in the immune response. Mutations in such genes have been shown to alter the immune cascade through recognition, presentation and elimination of pathogen by macrophages and neutrophils. The aim of our study was to determine if mutation in either of these genes increases the incidence of sepsis, prevalence to specific organism or alters the outcome of infection. This was examined in all states of neutrophil count.

We have selected to study two genes involved in innate immunity. The first gene is the human phagocyte-specific chitotriosidase. Increased enzyme activity has been demonstrated in patients with Gaucher disease,[100] and also reported in other lysosomal diseases.[118] Elevated activity was associated with gram-negative infection in children undergoing therapy for acute myeloid leukaemia suggesting

pleiotropic effects[96] and in neonates with fungal infection.[6] Other associations have been observed in malaria,[112] sarcoidosis,[121, 225] leprosy[226] visceral Leishmaniasis[112] and tuberculous pleural effusions.[227]

The second gene, NOD2 ,encodes the intracellular NOD2 receptor, expressed in the monocytes,[131] macrophages, dendritic cells,[144] intestinal epithelial[140] and Paneth cells.[145] which binds to MDP of bacterial cell wall and leading to inflammatory response.[93, 138, 139] Three alleles of NOD2 have been linked mainly to CD[4, 130] but also an association has been described with sepsis,[164, 175] sepsis related mortality in GvHD (hematopoietic stem cell transplant),[8, 9] small intestine allograft rejection[228] and more recently with spontaneous bacterial peritonitis in patients with cirrhosis.[229]

Chitotriosidase and NOD2 genes are both involved in the immune response by common recognition of pathogen molecules.[97] Molecules such as PGN which initiate the response by stimulating the neutrophils. Ejik et al even studied the effect of NOD2 activation on chitotriosidase expression by phagocytes.[97] After studying the effect of mutations in each gene separately we tried to find out if the combined mutation of both genes have a synergic effect on incidence of sepsis, prevalence to specific organism or alters the outcome of infection.The presence of mutations and the impact on the immune response in patients with haematological malignancy were the basis of our study. This impact was investigated in three different sub studies. The first sub study was carried out comparing the chitotriosidase genotypes (wild type, heterozygote and homozygote for the mutation). Secondly we compared NOD2 genotypes the wild type, the NOD2-SNP8; Arg702Trp and the NOD2-SNP12; Gly908Arg. The third sub study was comparative, carried out between four groups wild type for any mutation, chitotriosidase only mutation, NOD2 only mutation (SNP8 and SNP12) and combined chitotriosidase and NOD2 mutation.

7.1.1. Incidence and duration of neutropenia:

A reduction in neutrophil count is a usual finding in haematological malignancies and due to chemotherapy. The effect of neutropenia and duration of neutropenia on sepsis incidence, outcome and organism prevalence were studied in relation to gene status.

Analysis was carried out separately in the three sub studies. We compared the number of episodes of neutropenia per genotype and per patients. The incidence of neutropenia showed no statistical differences between the chitotriosidase or NOD2 genotypes. The finding was the same when mutation of either gene genotypes was studied or when the mutation of each gene was compared with the absence of mutation or the combined chitotriosidase NOD2 mutation.

There was no significant difference in the number of neutropenic episodes but there was a suggestion, which did not meet statistical significance, of an increase in duration of neutropenia with the homozygote chitotriosidase mutation. NOD2 genotypes did not appear to influence incidence or duration of neutropenia.

From the above findings we can conclude the absence of any relationship between innate immune genes, chitotriosidase and NOD2 (SNP8 & SNP12)) and neutrophils response to chemotherapy. There was no effect on the neutrophils fall or duration of regeneration in our study population of haematological malignancy patients regardless of genotype of chitotriosidase or NOD2.

7.1.2. Incidence of infective episodes:

Next we evaluated the incidence of sepsis in patients with haematological malignancy who were either neutropenic or not. To determine if mutation of two genes involved in innate immunity have any effect on neutrophil function we analysed the incidence of febrile events (infections) in relation to neutrophil according to genotype in each patient.

A febrile event was considered if temperature of 38.5°C or greater occurred once or 38°C in more than one occasion.We observed that the incidence of infection did not differ between chitotriosidase genotypes whatever the neutrophil count was. In contrast other studies have demonstrated a relationship between chitotriosidase and sepsis such as a study in children with the mutation undergoing AML chemotherapy.[96] Another study showed improved survival with the introduction of recombinant human chitotriosidase to neutropenic mice with *Aspergillosis*.[7] Explanations of our findings could be the wider variety of haematological malignancies, the patients' age (adults) and the species in which our study was conducted on (human *vs* mice).

The overall incidence of sepsis was the same for all three NOD2 genotypes (wild type NOD2, NOD2-SNP8; Arg702Trp or NOD2-SNP12; Gly908Arg). Although NOD2 response leads to NF-κB activation the degree of response to LPS and PGN differ between genotypes, due to selective defects in leukocyte function with NOD2 mutation.[230] The response in NOD2-SNP8; Arg702Trp and the NOD2-SNP12; Gly908Arg is diminished compared to the absence of response[230] in the more prevalent frameshift SNP13; L1007fsinsC.[4, 130] We could not examine this mutation due to technical difficulties (software and primer design).

The innate immune response in the low birth weight infants[163] and adults[164] has also been examined, in different studies and an increase incidence of sepsis was found in the mutant genotypes a finding we could not replicate in our study with adult patients.

NOD2 and the incidence of sepsis have been studied widely but not in relation to neutropenia. In our study the incidence of sepsis in NOD2 genotypes showed no significant association to neutrophil count. Similar studies are in post transplant (SCT) patients which showed increase TRM due to intestinal and pulmonary complications.[8, 9]

The incidence of sepsis was also not affected by combinations of the mutations of the innate immune genes (chitotriosidase and NOD2 (SNP8 & SNP12)).

From the above findings we can conclude the absence of any association between the incidence of sepsis whatever the neutrophil count was and any of the genes we looked at (NOD2 and chitotriosidase genotypes) in isolation or combination.

7.1.3.   Organism prevalence:

The final section of this study was to evaluate the relationship between mutations of (chitotriosidase and NOD2 (SNP8 & SNP12)) genes and prevalence of various organisms in patients with infective events. Organisms were divided into four groups; bacterial, fungal (proven and probable IFD), viral and unidentified (culture negative and possible IFD). Possible IFD is the term used when the radiology histopathalogy, cytology or fluid and body material direct examination are not suggestive of fungal infection despite the clinical picture.[183]   We felt that the evidence for fungal infection in these cases was so limited that it would be inappropriate to include these events in any group other than the culture negative.

Studies have shown increased chitotriosidase activity during bacterial infections in neonates[6] and immune compromised children due to AML and chemotherapy.[96]

Although we did not include measurements of chitotriosidase activity in our study, nevertheless we did find a positive association between bacterial cultures and heterozygote chitotriosidase mutations regardless of neutropenia.

This association arose in the presence of neutropenia for heterozygote chitotriosidase mutation. NOD2 mutations have also been observed in association with increased incidence of sepsis due to bacterial pathogens in both adults and neonates by different investigators.[163, 164] Although we did not identify the SNP13;L1007fsinsC which is considered to lack bacterial PGN/LPS response[230] and only identified the SNP8;Arg702Trp and SNP12;Gly908Arg, SNPs with decreased response to the same bacterial components[230] the NOD2 mutations showed significant association with bacterial infections in febrile events occurring in non-neutropenic patients. This finding was confirmed when the NOD2 mutation was considered in combination with the chitotriosidase.

Documented fungal infections (proven and probable) were so few in our study and we could not obtain a result in all the three sub studies. The diagnosis of fungal infection requires host factor, clinical and mycological criterion. Definite identification of fungal infection requires histological, cytopathalogical or direct microscopic examination of body material and fluids.[183] The absence of such mycological criterion even with the strong suspicions based on host-clinical factors make the category of diagnosis of fungal infection 'possible'. In our study we analysed the 'possible IFD' with the other culture negative events.

Documented viral isolates causing febrile events in neutropenic patients showed a statistical association with NOD2 mutations but with uncertain relevance since so few viral infections were confirmed. This may be because these organisms are difficult to identify technically (PCR not available for every virus), or loss of some patients from the study before samples were taken. We believe viral infections can be easily missed and miss-classified as culture negative. A larger study specifically of viral and fungal events would be necessary to confirm this finding.

The proportion of febrile events with negative cultures and so unidentified cause of infection was significantly higher in the chitotriosidase homozygote mutation genotypes. This significance was more pronounced with the presence of neutropenia.

As mentioned earlier the culture negative category includes possible fungal infections. Could this be the cause of more febrile events with unidentified cause of infections in the homozygote chitotriosidase mutation in our study? Keeping in mind the studies[7, 106, 182] mentioning the association of chitotriosidase and fungal infections.

Combining gene mutations showed significant association with FUO in relation to episodes of neutropenia, although none of the genes showed this association when studied separately. It looks from our comparative sub study that there is synergistic effect of combined mutations on susceptibility to bacterial infections even when there are adequate number of neutrophils. The synergistic effect is

more pronounced when the patient is neutropenic, as during episodes of neutropenia there were increase occurrences of FUO.

In conclusion it would appear that there were more bacterial isolates from patients with chitotriosidase mutations when neutropenic and more in patients with NOD2 genotypes or combined chitotriosidase and NOD-2 mutation when not neutropenic.

Culture negative infections were associated with both chitotriosidase genotypes and the combination of chitotriosidase and NOD2 during neutropenia. This association could be due to the synergistic effect of combined mutation on top of neutropenia. The question here is, does combined NOD2/chitotriosidase mutation predispose individuals in the set of immunosupression to infections with unusual organisms or does the patient immunological response alter the picture of clinical presentation!

Another question is how accurate is the diagnosis of culture negative fever is? Are we obtaining enough specimens or is technology here to blame?

The number of patients with combined mutations in our study was only 3.48% (7 out of 201 patients). The incidence of infections in the set of combined mutations involved in immune response may need to be investigated in wider scale of patients.

The outcome of sepsis was not significantly affected by any of the genes involved in our study. Severe infections and death has been investigated by various researchers and found to be increased in relation to NOD2 polymorphisms.[164, 175, 229]

The findings in our study could be of clinical significance in patients becoming immunocompromised in the course of their life due to disease or therapy (chemotherapy or SCT). As the innate immune mechanism is affected by mutations of chitotriosidase and NOD2, it might be possible to prevent or decrease the incidence of morbidity infections by taking extra prophylactic measures in patients known to have the relevant genotypes. Measures could be targeted according to mutation and Neutrophils status.

7.2.　　　NOD2 mutation and ALL outcome

Several studies[8, 9, 221] observed the effect of NOD2 polymorphisms on the outcome of SCT in immunocompromised patients and concluded an increase incidence in relapse and decrease overall survival. The incidence was higher when both recipient and donor carried the mutation. Contrary to this study Sairafi et al[10] showed no impact of NOD2 mutation in outcome post transplant. Similarly our study in ALL patients undergoing chemotherapy also showed no statistical significance of NOD2 polymorphisms and the outcome (CR, relapse or OS). Death was due to sepsis in 40% of our ALL population.

7.3.　　　Mutations:

7.3.1.　　　Chitotriosidase:

The frequency of chitotriosidase mutation was around 40.20%. The heterozygote gene mutation was 34.80% in our study population almost as same as the Portuguese population 30-40%,[107] and in the Netherlands among Ashkenazim[106] (37%). In both studies the homozygote mutation was about 6%,[106, 107] again close to 5.39% homozygote mutation seen in our study.

7.3.2.　　　NOD2:

There was a noticeable variation in NOD2 mutation frequencies in our two studies. This could be contributed to the different ethnic background and/or diagnosis. The NOD2 mutation frequency in our studies population was 13.31% (variation between the two studies; 8.46-34.04%). The Gly908Arg (SNP12) was the more frequent mutation.

The compound heterogeneity (SNP8 and SNP 12) mutation was 1.21% (variation; 1.0-2.13).

In the NOD2 mutation and ALL outcome study population, the three SNPs polymorphisms were successfully identified. The incidence of heterozygosity was 10.64%, 21.28% and 2.13% for Arg702Trp (SNP8), Gly908Arg (SNP12) and frameshift L1007fsins (SNP13) respectively. Homozygosity for the mutation was 2.13% and occurred only in the L1007fsins. The compound (Arg702Trp and Gly908Arg) allele mutation incidence was 2.13%.

In the genetic determinants of sepsis study the frequency of the heterozygote mutation was 3.48% and 5.57% for Arg702Trp (SNP8) and Gly908Arg (SNP12) respectively. Homozygosity for the mutation was 0.50% for Gly908Arg and compound heterozygosity for both SNPs was 1.0%. The Gly908Arg mutation occurred more frequently, while L1007fsins was the least identified mutation in this study. The frameshift L1007fsins identification was unsuccessful in this arm of the study. The initial genescanning technique failed as well as Anthony Nolan protocol[231] and even the pyrosequencing method used for detection of the two missense variants (Arg702Trp and Gly908Arg) did not work. Eventually we had to terminate our study exempting the frameshift mutation.

The three major NOD2 polymorphisms Arg702Trp (SNP8), Gly908Arg (SNP12) and the frameshift L1007fsins (SNP13) have demonstrated remarkable heterogeneity across ethnicities and populations with regional differences. The variations in the mutation frequency in our studies could be contributed to by the ethnic differences or the diagnoses. In the NOD2 mutation and ALL outcome study, patients were only diagnosed with ALL and the study was multicentre, raising the possibilities of differences in ethnicity eg variation in predominance of Caucasians in some parts of the UK [146]. In the genetic determinants of sepsis study, patients were diagnosed with various haematological malignancies (including AML, ALL, CLL, CML, MM... etc). An explanation for the low incidence of NOD2 mutation in this study population could be the mixture of diagnosis and ethnic background. For example Multiple myeloma incidence is as twice as common in patients of African descent compared to cauacasions[190] while NOD2 mutation is low in this group[147].

This study was only conducted in the RFH in central London with wide background ethnicities.

7.4.   Limitations:

Recruitment took place in the RFH between June 2006 - March 2008. Initially 310 patients with haematological malignancy were approached but only 257 consented. Out of the consented patients only 204 met the inclusion criteria.

The aim was to recruit 200-250 patients from in-patient haematological wards but later out-patient department was included due accelerate the recruitment process.

Blood samples were planned to be drawn with the routine investigations while in-patient admission. Later blood was collected from the haematology laboratory due to loss of in patient samples and convenience for patients 2-3 samples for each patient were required to reach the desired DNA concentration (200ng/µl), more samples were necessary when patients were neutropenic. Difficulties in obtaining enough blood were another obstacle in patients who had undergone allogenic transplant or patients who had died.

NOD2 identification was initially successful by gene scanning and results were obtained and checked randomly for accuracy by DNA sequencing. But this method was tricky and at a point stopped working although measures were taken to try and find out what went wrong. Measures such as; change of the primer set and annealing temperature (gradient temperature). Other methods such as NOD2 genotyping adopted by Anthony Nolan[231] was also tried but with no success. It took months – year to find a team to give us assistance and alternatively pyrosequencing was started with the help of Hammersmith haematology laboratory.

By pyrosequencing, SNP8 and SNP12 missense mutations were easily determined and distinguished from the wild type but this was different with SNP13 frameshift

mutation. Technical support advice was sought with no success. Unfortunately time limitations meant the laboratory work had to be completed without SNP13.

Identification of NOD2 SNPs 8 and 12 by genescanning and pyrosequencing were compared for the same 50 DNA samples. The results obtained were identical, confirming the equality and accuracy of both methods used.

7.5.   Conclusions:

Polymorphisms in genes involved in innate immunity appear to be associated with bacterial infections. Chitotriosidase mutations were associated with bacterial infections in the neutropenic patients while NOD2 and combinations of mutations were similarly associated in the non-neutropenic patients.

Innate immune gene polymorphisms also seem to predispose to infections with unidentified pathogens in the presence of neutropenia. This association was seen in patients with the homozygote mutation of chitotriosidase and the combination of NOD2 and chitotriosidase mutations.

Documented viral infection was associated with NOD2 mutations in the presence of neutropenia, but in a very small number of patients.

Appendix-1

A.1. Recruitment forms

A.1.1.   Invitation letter

Study title:
Genetic determinants of sepsis and gasterointestinal pathology in haematological malignancy

Dear Patient

You are invited to join our study of genetic determinants of sepsis and gasterointestinal pathology in haematological malignancy, as you did fulfill our inclusion criteria. If you do wish to participate please read the information leaflet and sign the consent form attached. Should you want to discuss any aspect of the study we will be happy to explain it.

Thank you

Research Fellow
Lamya Saeed

Study Number:
Patient Identification Number for this study:

A.1.2. CONSENT FORM

Title of Project:
Genetic determinants of sepsis and gastrointestinal pathology in haematological malignancy

Name of Researcher:
Dr Satish Keshav MBBCh DPhil FRCP, Consultant Physician & Gastroenterologist
Dr Lamya Saeed
Dr Derralynn Hughes
Professor S MacKinnon
Professor Alejandro Madrigal

Please initial box

1. I confirm that I have read and understand the information sheet dated .... ☐
    for the above study. I have had the opportunity to consider the information,
    ask questions and have had these answered satisfactorily.

2. I understand that my participation is voluntary and that I am free to withdraw .... ☐
    at any time, without giving any reason, without my medical care or legal rights
    being affected.

3. I understand that relevant sections of any of my medical notes and data collected .. ☐
    during the study, may be looked at by researchers involved in the study.
    I give permission for these individuals to have access to my records.

4. I agree to my GP being informed of my participation in the study. ☐

5. I agree to take part in the above study. ☐

_____   _____
Name of Patient   Date   Signature

_____   _____
Name of Person taking consent   Date   Signature
(if different from researcher)

_____   _____
Researcher   Date   Signature

When completed, 1 for patient; 1 for researcher site file; 1 (original) to be kept in medical notes

A.1.3. Patient Information Sheet

Part 1.

Study title: Genetic determinants of sepsis and gastrointestinal pathology in haematological malignancy.

This information sheet is written to help to answer questions you might have about this study, which we have invited you to participate in. In addition to reading this, you will have the opportunity to ask questions from the researchers in person, and you should participate only if you fully understand what we are proposing.

What is the purpose of the study?
To find out what influences the chances of developing an infection as a consequence of having leukaemia, lymphoma, or a number of other blood disorders, particularly after treatment with anti-cancer drugs. Specifically, we want to understand the influence of inherited genes, such as the NOD2, Chitotriosidase, and Dectin genes, on the susceptibility to and severity of infections. Also there is some evidence of NOD2 involvement in other conditions.

Why have I been chosen?
Patients suffering from blood cancers are prone to severe infections, and as you are in this category, we want to study if you have any of the genes that we think may make patients with blood cancers prone to severe infections.

Do I have to take part?
Participation is completely voluntary, and if you do not feel able to participate this will not effect your treatment or care in any way.

What will happen to me if I take part?
A small tube of blood (approximately x tablespoon/10mls) will be taken in addition to your usual blood tests, and during your stay in hospital, we will keep a check on your blood count, and whether you develop a fever or infection. We will take blood when most convenient for you e.g. at the time when other bloods are taken as part of your standard hospital care.

The blood sample will be used to test your DNA for the presence of particular genes.
You will not be required to do anything else, and your medical treatment will not be influenced in any way.
If you leave hospital while still on antibiotics, we will check on your condition when you come back to clinic.

What do I have to do?
All you need to do is to give a blood sample, for DNA extraction and analysis. If you wish to take part in this study you will be given a copy of this information sheet and a signed consent form to keep.

What are the risks of taking part?
There are no risks to you.

What are the benefits of taking part?
By finding out the relation between infections and NOD2, Chitotriosidase, and Dectin genes status, we might be able to prevent fever and infections in certain patients in the future, and thus improve treatment. However, it is unlikely that this research will benefit you personally.

What if relevant information becomes available?
Sometimes during the course of a research project, new information becomes available about the area that is being studied. If this happens, we will tell you about it and discuss whether you wish to participate.

What happens when the research study stops?
The information will be analysed and stored. Your name and identity will remain anonymous.

What if there is a problem?
You will be able to contact any of the study staff, and they will liaise with your regular medical carers.

If you have a concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your questions (Dr Lamya Saeed, 32887; Dr Satish Keshav, ext 32882; Derralynn Hughes, bleep 71-1720). If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the hospital.

In the event that something does go wrong and you are harmed as a result of taking part in this research study there are arrangements for non-negligent harm/no-fault compensation. If you are harmed and this is due to someone's negligence then you may have grounds for a legal action for compensation but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you.

Will information about me be kept confidential?
Yes. All the information collected about you during the course of the study will be strictly confidential. Any published report or research will not identify you.

This completes Part 1 of the information sheet.
If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making any decision.

Part 2

What will happen if I do not want to carry on with the study?
Participation in the study is entirely voluntary, withdrawal from the study will not affect your medical care and your data and samples can be destroyed if you request this.

Will my taking part in this study be kept confidential?
Yes. All information which is collected about you during the course of research will be confidential. Name, address and records identification will be removed and data will have a number and stored in excel sheet.

What will happen to any sample I give?
Blood will be extracted only once in the beginning of the participation and used for DNA extraction and genotyping.

Will any genetic tests be done?
Yes. We will look for the presence of particular genes such as the NOD2, Chitotriosidase, and Dectin on the susceptibility to and severity of infections.

What will happen to the results of the research study?
The research results will be reported in the scientific and medical literature. You will not be identified in any report/publication. If you wish to be informed of the study's finding, please inform Dr Keshav or Dr Saeed.

Who is organizing and funding the research?
This study is the initiative of researchers in the departments of Gastroenterology and Haematology, and the funding is from various sources. If the study results are positive, further funding from public bodies will be sought. University College London (UCL) is the Research Governance sponsor for this study

Who has reviewed the study?
The study has been reviewed by scientific and medical colleagues who are not connected with the study, and by the Ethics committee of the Royal Free Hospital.

Contacts: Dr Lamya Saeed, ext. 32887, Dr Satish Keshav, ext. 32882, Derralynn Hughes, bleep 71-1720

A.1.4. GP letter

Date

Dr General Practioner
Community Health Centre
Street
Suburb
City
Postcode
Country

Dear Dr

<u>RE: Name</u>
<u>DOB</u>
<u>Hosp. no</u>

Your patient has kindly agreed to participate in our study "Genetic determinants of sepsis in haematological malignancy".

The study will involve drawing 10ml of blood during routine venepuncture for DNA extraction and subsequent genotyping. During the patient's stay in hospital a data sheet will be maintained charting their WBC count and details of any septic episodes. Patients will be followed in their in-patient stay and the outcome of their stay will be recorded. Longer follow up may be needed in a few patients, who are discharged whilst still on antimicrobial therapy.

We do not anticipate that participation will have any effect on their treatment or its outcome. If you have any questions relating to this study, please contact us.

Yours sincerely

Dr Satish Keshav   Dr L. Saeed
Senior lecturer & Consultant Physician   Clinical research fellow
Gastroenterology   Gastroenterology

cc: Dr Derralyn Hughes, Lecturer in haematology
cc: Professor Stephen MacKinnon, …
cc: Professor Jose Alejandro Madrigal, ...

Appendix-2

A.2. Follow up forms

A.2.1. NOD-2 and Sepsis project
Back ground haematology history

Name:
DOB: Hosp Number:
Date admitted: Date assessed:

Diagnosis on admission:
Date first diagnosed:
Place of diagnosis:

Referring GP & address:

1st line therapy:
Response:
Date of 1st CR:
Duration of CR:
Date of 1st relapse:

2nd line therapy:
Response:
Date of 2nd CR:
Duration 2nd CR:
Date of 2nd relapse:

Background & follw up:

Summary:
Admission

Neutropenia (/7)

Neutropenic episode=
Neutropenic sepsis=

Status on D/C *Neutopenia*
                    Antibiotics
                    Prophylactic regimen

A.2.2. Data collection sheet page 1

Study: Genetic determinants of sepsis in haematological malignancy

Table 1 – Basic data

Case number:		Hospital number:		Date of birth:	Sex: M *or* F
Diagnosis:		BMT? N *or* A *or* R		Date of admission:	Date of discharge:
Neutropenia: Y *or* N		Sepsis: N *or* Y ? clinical *or* proven		Discharge status:	
DNA sample		NOD2		Chitotriosidase	Dectin

Table 2 – Daily data during admission

Day No.		1	2	3	4	5	6	7
Date								
WCC (10^9/ml)								
PMN (10^9/ml)								
Max temp (°C)								
Min temp (°C)								
Not septic								
?Septic								
Clinical sepsis								
Proven sepsis								
Culture result								
Site of sepsis								
Antibiotics								
Antifungals								
Other:								
Day No.		8	9	19	11	12	13	14
Date								
WCC (10^9/ml)								
PMN (10^9/ml)								
Max temp (°C)								
Min temp (°C)								
Not septic								
?Septic								
Clinical sepsis								
Proven sepsis								
Culture result								
Site of sepsis								
Antibiotics								
Antifungals								
Other:								

Continue notes on back of sheet if necessary.   For hospital stay >14 days, use continuation sheets.

Table 3 – Notes (with dates):

Sepsis:	Culture:
Antimicrobial treatment:	Imaging/radiology:
Relevant haematology/comorbidity:	Other: G-CSF? GM-CSF?

For hospital stay >14 days, use continuation sheets.

Data collection sheet page 2

Study: Genetic determinants of sepsis in haematological malignancy

Table 1 – Basic data

Case number:		Hospital number:		Date of birth:		Sex: M *or* F	
Diagnosis:		BMT? N *or* A *or* R		Date of admission:		Date of discharge:	
Neutropenia: Y *or* N		Sepsis: N *or* Y ? clinical *or* proven		Discharge status:			
DNA sample		NOD2		Chitotriosidase		Dectin	

Table 2 – Daily data during admission

Day No.		15	16	17	18	19	20	21
Date								
WCC (10^9/ml)								
PMN (10^9/ml)								
Max temp (°C)								
Min temp (°C)								
Not septic								
?Septic								
Clinical sepsis								
Proven sepsis								
Culture result								
Site of sepsis								
Antibiotics								
Antifungals								
Other:								
Day No.		22	23	24	25	26	27	28
Date								
WCC (10^9/ml)								
PMN (10^9/ml)								
Max temp (°C)								
Min temp (°C)								
Not septic								
?Septic								
Clinical sepsis								
Proven sepsis								
Culture result								
Site of sepsis								
Antibiotics								
Antifungals								
Other:								

Continue notes on back of sheet if necessary.   For hospital stay >28 days, use continuation sheets.

Table 3 – Notes (with dates):

Sepsis:	Culture:
Antimicrobial treatment:	Imaging/radiology:
Relevant haematology/co-morbidity:	Other: G-CSF? GM-CSF?

For hospital stay >14 days, use continuation sheets.

Data collection sheet page __

Study: Genetic determinants of sepsis in haematological malignancy

Table 1 – Basic data

Case number:		Hospital number:		Date of birth:		Sex: M *or* F	
Diagnosis:		BMT? N *or* A *or* R		Date of admission:		Date of discharge:	
Neutropenia: Y *or* N		Sepsis: N *or* Y ? clinical *or* proven		Discharge status:			
DNA sample		NOD2		Chitotriosidase		Dectin	

Table 2 – Daily data during admission

Day No.		__	__	__	__	__	__	__
Date								
WCC (10^9/ml)								
PMN (10^9/ml)								
Max temp (°C)								
Min temp (°C)								
Not septic								
?Septic								
Clinical sepsis								
Proven sepsis								
Culture result								
Site of sepsis								
Antibiotics								
Antifungals								
Other:								
Day No.		__	__	__	__	__	__	__
Date								
WCC (10^9/ml)								
PMN (10^9/ml)								
Max temp (°C)								
Min temp (°C)								
Not septic								
?Septic								
Clinical sepsis								
Proven sepsis								
Culture result								
Site of sepsis								
Antibiotics								
Antifungals								
Other:								

Continue notes on back of sheet if necessary.   For longer hospital stay, use continuation sheets.

Table 3 – Notes (with dates):

Sepsis:	Culture:
Antimicrobial treatment:	Imaging/radiology:
Relevant haematology/co-morbidity:	Other: G-CSF? GM-CSF?

For hospital longer hospital stay, use continuation sheets.

Appendix-3

A.3.

WHO Classification of neoplastic diseases of the haematopoietic and lymphoid tissues

I. Proposed WHO classification of myeloid neoplasms

1- Chronic Myeloproliferative Diseases (MDP)*
Ch myelogenous leukaemia, Philadelphia chromosome positive (t(9;22)(qq34;q11), BCR/ABL)
Ch neutrophilic leukaemia
Ch eosinophilic leukaemia/hypereosinophilic syndrome
                     Male : Female-9:1
Ch idiopathic myelofibrosis
Ch myeloproliferative disease, unclassified
Polycythemia vera
Essential thrombocythemia

2- Myelodysplastic / Myeloproliferative Diseases*
Ch myelomonocytic leukaemia (CMML)
Atypical ch myeloid leukaemia (aCML)
Juvenile myelomonocytic leukaemia (JMML)
Myelodysplastic/myeloproliferative diseases, unclassifiable

3- Myelodysplastic Syndromes*
Refractory anaemia (RA)
        -with ringed sideroblasts (RARS)
        -without ringed sideroblasts
Refractory anaemia with excess blasts (RAEB)
Refractory cytopenia with multilineage dysplasia (RCMD)
Myelodysplastic syndrome
        -associated with isolated del (5q) chromosome abnormality
        -unclassifiable

4- Acute Myeloid Leukaemia*
AML with recurrent cytogenic abnormalities
        -AML with t(8;21)(q22;q22), AML1(CBFα)/ETO)
        -AML with abnormal bone marrow eosinophils (inv(16)(p13q22)) or t(16;16)(p13;q22), (CBFβ/MYH11)
        -Acute promyelocytic leukaemia (AML with t(15;17)(q22;q11-12), (PML/RARα) and variants)
        -AML with 11q23 (MLL) abnormalities

AML with multilineage dysplasia
        -with prior myelodysplastic syndrome
        -without prior myelodysplastic syndrome

AML and myelodysplastic syndromes, therapy related
        -Alkylating agent rlated
        -Topoisomerase II inhibitor-related (epipodophyllotoxins, etoposide)
        -Other types

AML not otherwise categorized
        -AML minimally differentiated
        -AML without maturation
        -AML with maturation
        -Acute mylemonocytic leukaemia
        -Acute monoblastic and monocytic leukaemia
        -Acute erythroid leukaemia
        -Acute megakaryoblastic leukaemia
        -Acute basophilic leukaemia
        -Acute panmyleosis with myelofibrosis
        -Myloid sarcoma

Acute leukaemia of ambiguous lineage
        -Acute biphenotypic leukaemias

II.   Proposed WHO classification of lymphoid neoplasms

1- B-Cell Neoplasms
   Precursor B-cell neoplasm*
        -Preccursor B lymphoblastic leukaemia/lymphoma

   Mature (peripheral) B-cell neoplasms*
        -Ch lymhocytic leukaemia/small lymphocytic lymphoma
        -B-cell prolymphocytic leukaemia
        -Lymphoplasmacytic lymphoma
        -Splenic marginal zone lymphoma (+/- villous lymphocytes)
        -Hairy cell leukaemia
        -Plasma cell myeloma / plasmacytoma
        -Solitary plasmacytoma of bone
        -Extraosseous plasmacytoma
        -Extanodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT-lymphoma)
        -Nodal marginal zone B-cell lymphoma (+/- monocytoid B cells)
        -Follicular lymphoma
        -Mantle cell lymphoma
        -Diffuse large B-cell lymphoma
            -Mediastinal (thymic) large B-cell lymphoma
            -Intravascular large B-cell lymphoma

-Primary effusion lymphoma
-Burkitt lymphoma/Burkitt's cell leukaemia

B-cell proliferations of uncertain malignant potential**
-Lymphoid granulomatosis
-Post-transplant lymphoproliferative disorder, polymorphic

2- T-Cell And NK-Cell Neoplasms

Precursor T-cell neoplasms*
-Precursor T lymphoblastic leukaemia/lymphoma
-Blastic NK cell lymphoma***

Mature T-cell and NK-cell neoplasms*
-T-cell prolymphocytic leukaemia
-T-cell large granular lymphocytic leukaemia
-Aggressive NK cell leukaemia
-Adult T-cell leukaemia/lymphoma (HTLV1+)
-Extranodal NK/T-cell lymphoma, nasal type
-Enteropathy-type γ☐ T-cell lymphoma
-Hepatosplenic T-cell lymphoma
-Subcutaneous panniculitis-like T-cell lymphoma
-Mycosis fungoides
-Sezary syndrome
-Primary cutaneous anaplastic large cell lymphoma (T/null cell)
-Peripheral T-cell lymphoma, unspecified
-Angioimmunoblastic T-cell lymphoma
-Anaplastic large cell lymphoma (T/null cell, primary systemic)

T-cell proliferation of uncertain malignant potential**
-Lymphoid papulosis

3- Hodgkin Lymphoma*
Nodular lymphocyte predominant HL
Classical HL
Nodular sclerosis classical HL
Lymphocyte-rich classical HL
Mixed cellularity classical HL
Lymphocyte-depleted classical HL

III.   Histiocytic And Dendritic-Cell Neoplasms

1- Macrophage/histiocytic neoplasm*
Histocytic sarcoma

2- Dendritic cell neoplasms
Langerhans cell histocytosis**

Langerhans cell sarcoma*
Interdigitating dendritic cell sarcoma*/tumour**
Follicular dendritic cell sarcoma*/tumour**
Dentritic cell sarcoma, not otherwise specified*

1V.   Mastocytosis; Mast Cell Diseases

Cutaneous mastocytosis
Indolent systemic mastocytosis**
Systemic mastocytosis with associated
         clonal, haematological non-mast cell lineage disease*
Aggressive systemic mastocytosis*
Mast cell leukaemia*
Mast cell sarcoma*
Extracutaneous mastocytoma**

Behave as malignant tumours, *
Lesions of low or uncertain malignant potential, **
Neoplasms of uncertain lineage and stage of differentiation, ***

1. NL Harris, ES Jaffe, J Diebold, G Flandrin, HK Muller-Hermelink, J Vardiman, TA Lister & CD Bloomfield. The world Health Organizaton classification of neoplastic diseases of the haematopoietic and lymphoid tissues: report of the Clinical Advisory Committee Meeting, Airlie House, Virginia, November 1997. Histopathalogy. 2000, 36, 69-86.
2. ES Jaffe, NL Harris, H Stein, JW Vardiman. World Health Organization Classification of Tumours. Pathalogy & Genetics. Tumours of Haematopoietic and Lymphoid Tissues. IARC Press: Lyon2001.

Appendix-4

A.4. RFH Policy

A.4.1.

Anti – Infective Therapy Guidelines For Patients with Haematological Disease.

ADULT EDITION

Updated and Amended in 2008

by:
Dr Archie Prentice, Prof Chris Kibbler,
Prof Paul Griffiths, Dr Ron Chakraverty, Hannah Kershaw,
Dr Sara Ghorashian and Dr Ben Killingley

CONTENTS 2008 VERSION

INTRODUCTION

There are two sets of anti-infective therapy guidelines for patients with a haematological disease, this one for adults and another one for paediatrics (Supportive care protocols paediatric haematology and oncology – found in the guidelines section on Freenet).

The aims of the guidelines are to ensure that anti-infective treatment with an appropriate spectrum of activity is given and to avoid over prescribing. As with all protocols, specific clinical situations may dictate modifications, and we have attempted to include some general principles to help in these situations. The majority of the recommendations are for empirical treatment of infections. If a specific microbiological diagnosis is made, treatment may require modification.

These guidelines are specially tailored to the requirements of a particular group of patients, i.e. haematological oncology patients. They are, therefore, not directly applicable to other patients on cytotoxic regimens or with neutropenia.
Patients eligible for ongoing clinical trials should, wherever possible, be entered into trials rather than following this protocol.

Further details of drugs and doses are included in Section 4.

If you are not experienced in the management of neutropenic and HSCT patients, the management of febrile patients should always be discussed.

Advice may be sought from: -
- Haematology Consultants and SpRs
- Microbiology Registrar - Bleep 1907 or Ext. 33973
- Virology Registrar - Bleep 1669 or Ext. 34089
- Haematology Pharmacists – Bleep 1873, 1341 and 2054
- On-call Pharmacist via switchboard out of hours
- Out of hours the microbiology registrar on call can be contacted via bleep 1710 or via switchboard

Royal Free Hospital: 020 7794 0500

1. NEUTROPENIC AND HSCT PATIENTS

1.1. PROPHYLAXIS

1.1.1 Standard Regimen (NB Note HSCT amendments)

Prophylaxis should be given to all patients receiving myelosuppressive therapy, i.e. any block of chemotherapy that will produce a prolonged period (>7 days) of profound neutropenia (neutrophils < 0.5 x 10^9/l).

Patients who had prolonged (>7 days) periods of neutropenia (< 0.1 x 10^9/l) on previous courses of chemotherapy normally considered non-myelosuppressive, should receive standard prophylaxis for subsequent courses e.g. CHOP, CODOX-M.

Standard Regimen:

- Chlorhexidine (0.2%) mouthwash - 5ml four times a day

- Aciclovir – HSCT patients only; for those who are HSV IgG antibody positive, give 400 mg po tds for seven days or until engraftment

- Also see fungal protocol

Commencing prophylaxis:
Standard prophylaxis should be commenced on the first day of chemotherapy. Antifungal prophylaxis should, where possible, be commenced the day before chemotherapy. **However, itraconazole must not be given until 24 hrs after cylophosphamide conditioning has been administered. Itraconazole must not be used at all in patients on vincristine – see antifungal policy.**
If the patient is neutropenic on presentation (neutrophils <0.5 x 10^9/l) prophylaxis should be commenced immediately.

Stopping prophylaxis:
Prophylaxis can be stopped when neutrophils are > 0.5 x 10^9/l on 2 consecutive readings within 3 to 4 days.

1.1.2. Specific Prophylaxis

CMV Prophylaxis
Prophylaxis is no longer used to prevent CMV infections after HSCT, instead pre-emptive therapy is used (see next section). Patients receiving highly immunosuppressive chemotherapy, such as Fludarabine, 2cDA or CAMPATH may also be considered for CMV pre-emptive therapy.

Endocarditis prophylaxis
Please see below. In almost every situation prophylaxis is not indicated.
Please discuss with microbiology if there are any queries.

NICE guidance

Antimicrobial prophylaxis against infective endocarditis in adults and children undergoing interventional procedures (March 2008)

Antibacterial prophylaxis and chlorhexidine mouthwash are not recommended for the prevention of endocarditis in patients undergoing dental procedures.

Antibacterial prophylaxis is not recommended for the prevention of endocarditis in patients undergoing procedures of the:
  – upper and lower respiratory tract (including ear, nose, and throat procedures and bronchoscopy);
  – genito-urinary tract (including urological, gynaecological, and obstetric procedures);
  – upper and lower gastro-intestinal tract.

Whilst these procedures can cause bacteraemia, there is no clear association with the development of infective endocarditis. Prophylaxis may expose patients to the adverse effects of antimicrobials when the evidence of benefit has not been proven.

Any infection in patients at risk of endocarditis should be investigated promptly and treated appropriately to reduce the risk of endocarditis.

If patients at risk of endocarditis are undergoing a gastro-intestinal or genito-urinary tract procedure at a site where infection is suspected, they should receive appropriate antibacterial therapy that includes cover against organisms that cause endocarditis.

Patients at risk of endocarditis should be:
  – advised to maintain good oral hygiene;
  – told how to recognise signs of infective endocarditis, and advised when to seek expert advice.

GVHD or Hyposplenism
Penicillin V 500mg bd po (if not on co-trimoxazole). If penicillin allergic, use Erythromycin 250mg bd po. Continue lifelong. Vaccinate with Pneumovax,Hib vaccine, and Meningococcal C vaccine at least two weeks before an elective splenectomy or as soon as possible before an emergency procedure.

Herpes simplex virus prophylaxis
Chemotherapy patients with IgG antibodies to HSV present should be prescribed Aciclovir po 400 mg tds while neutropenic. Patients who are negative for HSV antibody should stop taking aciclovir. HSCT patients with IgG antibodies to HSV presenting pre-transplant should be prescribed aciclovir po 400mg tds for 100 days from transplant. If the patient is unable to tolerate the oral route, give aciclovir iv 5mg/kg tds.

Pneumocystis jiroveci (PCP)

The following groups of patients should receive prophylaxis with co-trimoxazole 960mg 3x a week (Mon, Wed, Fri)[1].

- Allogeneic HSCT patients (see below)
- Patients receiving fludarabine or cladribine (continue for 6 months post last dose)
- Patients receiving > 40 mg prednisolone po daily for > 2 weeks[2]
- Patients with ALL for 120 weeks in continuous complete remission or at least 1 extra year after any extramedullary relapse[3]
- Hodgkins Disease

Allogeneic HSCT Patients:-

During the week prior to BMT, give co-trimoxazole 960mg bd po daily until day -1 of the conditioning regimen. In the immediate post transplant period give nebulised pentamidine 150mg every two weeks (after nebulised or inhaled salbutamol) or 300mg monthly until engraftment. In the first three months post transplant, co-trimoxazole is especially associated with marrow toxicity. Restart co-trimoxazole at a dose of 960mg bd po on 2 consecutive days per week when counts have recovered (neutrophil count > 1.0 x 10^9/l) and continue for at least six months post transplant, or until CD4 count is > 200.

Autologous HSCT patients only require co-trimoxazole if previously treated with a purine antagonist as above

If allergic to co-trimoxazole: prescribe dapsone po 100mg once daily (check G6PD where appropriate).

1 Green H, Paul M, Vidal L, Leibovici L. Prophylaxis for Pneumocystis pneumonia (PCP) in non-HIV immunocompromised patients. Cochrane Database Syst Rev. 2007 Jul 18;(3)
2 Yale SH, Limper AH. Pneumocystis carinii pneumonia in patients without acquired immunodeficiency syndrome: associated illness and prior corticosteroid therapy. Mayo Clin Proc. 1996 Jan;71(1):5-13.
3 Hughes WT, Rivera GK, Schell MJ, Thornton D, Lott L. Successful intermittent chemoprophylaxis for Pneumocystis carinii pneumonitis. N Engl J Med. 1987 Jun 25;316(26):1627-32.

Toxoplasmosis

If the stem cell donor or recipient has evidence of a recent clinical infection (IgM ELISA positive) give the following for 6 weeks;
- Pyrimethamine 100 mg po twelve hourly on day 1 as a loading dose, then 25 mg po tds
- Sulfadiazine 3g po as loading dose then 2g po tds, and calcium folinate 15mg po od

Tuberculosis

Give prophylaxis if past history of TB or suspicious CXR, irrespective of geographical background. Otherwise see Trust Guidelines for TB prophylaxis in immunocompromised patients. Usual regimen = isoniazid 300mg po od and pyridoxine 25mg po od for 6 months after HSCT or for duration of neutropenia. Remember to monitor LFT's closely and to discontinue treatment if significant deterioration occurs.

Viral Contact

- Chickenpox or zoster: Check IgG status to VZV. If negative, give Zoster Immunoglobulin (ZIG) and consider oral Valaciclovir 1g tds from day 7-14 (where day of first contact is day 0)

- Measles: Normal immunoglobulin, one dose for an adult is 750mg im· If patient is thrombocytopenic give a stat dose of Immunoglobulin iv 400mg/kg and repeat if further exposure occurs after 3 weeks

- Parvovirus and other exanthemata: discuss with Virology Department.

1.2 PRE-EMPTIVE TREATMENT

1. *Pseudomonas aeruginosa* in neutropenic patients
(isolated from mucosal sites e.g. stool, mouth)
Discuss with microbiology, options include ciprofloxacin, aztreonam, ceftazidime, piptazobactam and meropenem.

2. *Staphylococcus aureus* in neutropenic patients
If present on nasal swab use topical Mupirocin intranasally tds for 5 days. MRSA eradication should be carried out using the standard protocol (infection control policies available on Freenet)

3. Galactomannan in High Risk Patients
Patients at increased risk of invasive fungal infection should be screened twice a week for invasive aspergillosis with serum galactomannan. Empiric therapy may be commenced if two consecutive samples are positive (after discussion with microbiology). Patients at increased risk of invasive fungal infection include adults on therapy for ALL, AML, allogenic transplant patients, patients on high dose cytarabine (ara-c) and those with severe GVHD (see antifungal protocol for details).

4. CMV surveillance in HSCT patients
Quantitative CMV PCR should be requested on a regular basis in HSCT patients (send a citrated blood sample on Tuesdays and Thursdays).
For patients with matched unrelated donors who are CMV seronegative (and so will not have adoptively transferred some immunity to the recipient), pre-emptive treatment is warranted if a single CMV PCR result is positive. For the remaining patients,pre-emptive treatment is warranted if the viral load is 3000 genomes/ml or greater in a single sample.
A number of ongoing clinical trials are performed at the Royal Free Hospital in relation to pre-emptive therapy for CMV. Please consult with the virology team and, where appropriate, the relevant trial protocol.

Pre-emptive therapy consists of ONE of the following along with G-CSF (lenograstim) 263 microgram subcut / iv once daily, to be initiated if neutrophils <1.0 x 10^9/l and continued according to response.

First Line therapy:
Ganciclovir 5 mg/kg iv bd (reduced with renal impairment)

Other therapeutic options include:

- Foscarnet 90mg/kg iv bd (reduced with renal impairment - see appendix for dosing guidelines and discuss with specialist pharmacist). This is to be used in preference to ganciclovir if the patient is neutropaenic
- Ganciclovir 5 mg/kg iv od PLUS Foscarnet 90mg/kg iv od
- Valganciclovir 900mg po bd (reduced with renal impairment) Valganciclovir is usually well-absorbed, even in patients who have GvHD affecting the gut, but monitor twice-weekly PCR results carefully and consider swopping to iv ganciclovir if there is any doubt about absorption. Valganciclovir may be used in low-risk patients with low CMV loads after discussion with the attending haematology consultant, and a member of the virology team.

Patients with >3000 genomes/ml stop treatment when two consecutive viral load results are negative or according to trial protocol as appropriate.
Patients treated because their viral load is below 3000 on two consecutive samples should stop treatment when two consecutive negative PCR values have been obtained.

5. HHV- 6 in HSCT patients
HHV-6 PCR should be performed on both donor and recipient pre-transplant to detect individuals with integrated DNA.

1.3 EMPIRICAL TREATMENT OF FEVER IN NEUTROPENIC PATIENTS

Patients with fever of 38.5^{o}C on one occasion should receive prompt empirical antibiotic therapy. Patients with a temperature of 38°C should be checked again in 1 hour and if still 38°C, or above, should receive prompt empirical antibiotic therapy. Use of antipyretic therapies (e.g. steroids) should be taken into account as should the normal temperature or even hypothermia in a septic patient. Conversely, other non-infective causes of fever must be considered (e.g. blood and drugs).

Initial Investigations: at least two sets of blood cultures from two separate sites, CXR, urine, as well as specimens from clinically suspicious sites.

If patient is on oral itraconazole prophylaxis then an itraconazole level should be measured. Obtain a trough level by taking a blood sample immediately prior to the next dose of itraconazole after starting the antibiotics. It can take up to 3 days for results to become available and this will be used to guide the empirical anti fungal therapy after 96hrs. Galactomannan can also be requested on this sample.

Examination of febrile neutropenic patients must include:
Specific questions as to the presence of;
- respiratory tract symptoms – nasal congestion, sore throat, cough, pleuritic chest pain, shortness of breath
- painful ulcers/lesions in the groin/perineum
- new rash, or new onset of diarrhoea

Full examination including the skin and mucous membranes, as well as the perineum and peri-anal area. Fundoscopy can reveal systemic infection (eg candida endophthalmitis). This must be repeated daily whilst the fever is ongoing

Repeat blood cultures every 24 hours while febrile (>38°C).
Arrange CT scanning of the chest if fever persists >96 hours

If pulmonary infiltrates are present on plain chest radiography with a non-productive cough, an early BAL should be arranged in addition to the described schedule. (See section 1.4.4 for HSCT patients with pneumonitis). The algorithm below presumes the patient is receiving antifungal prophylaxis.

Note there is no evidence for the addition of teicoplanin in the absence of clinical signs of line infection or positive cultures, nor is the empiric addition of amikacin warranted in patients with continuing fever unresponsive to meropenem.
If the patient remains hypotensive and shocked despite aggressive fluid resuscitation and oxygen therapy, the addition of amikacin should be discussed with the consultant haematologist on call and microbiology.

Algorithm for the Antibiotic Management of Febrile Neutropenia in Haematology Oncology Patients

PMN < 0.5 x 10^9/1
Temp. > 38.5°C
or 38°C x2

Eligible for current trial? — **YES** → Follow protocol

↓ NO

Start Meropenem

Deterioration
- fall in BP at anytime — **YES** → Resuscitate
Repeat cultures & CXR. Discuss addition of Amikacin

↓ NO

Significant isolate? — **YES** → Modify antimicrobials if appropriate

↓ NO

Pulmonary infiltrates? — **YES** → See Section 1.4.3 - fever with pulmonary infiltrates

↓ NO

Other clinical site of sepsis? e.g. Hickman site — **YES** → Investigate and treat as appropriate

↓ NO

Response by 96 hours? — **YES** → Continue for 7 days

↓ NO

Do chest CT if not already done
- consider trial
- see empirical antifungal protocols

Response by 96 hours? — **NO** → Do chest CT

↓ YES

Continue

213

1.4 INFECTIONS IN NEUTROPENIC PATIENTS

1.4.1 Notes On Therapy

1. Definite Bacteraemia.
(Two or more sets of blood cultures positive for the same organism.)
When a definite bacteraemia has been diagnosed, antibiotic therapy should be rationalised (which may include continuation of broad spectrum cover). When a Gram positive infection is diagnosed Gram negative cover with non-aminoglycoside monotherapy should be continued for five days. Methicillin resistant coagulase negative staphylococcal bacteraemia requires treatment with teicoplanin.

2. Persistent Fever.
If a definite infection fails to respond to appropriate therapy the most likely causes are:
– Fungal infection - consult the anti-fungal policy.
– An undetected focus of infection (including line sepsis).
– In the absence of a source of infection, consider removal of the Hickman line.

3. Allergy.
If ANAPHYLAXIS has occurred with one class of beta-lactam antibiotic, the use of others is contra-indicated. Use teicoplanin, amikacin, and initiate ciprofloxacin intravenously.
With beta-lactam allergy (e.g. rash), use meropenem (there is a low risk of cross reactivity of carbapenems with other beta-lactams)

4. Duration.
– Antibiotic treatment should be given for at least seven days with an apparently effective antibiotic, with at least four days without fever. Patients whose neutrophil counts have recovered should be individually assessed.
– If fever has responded to empirical anti-fungal therapy, this should be continued at least until the neutrophil count is $\geq 0.5 \times 10^9$/l. Refer to the anti fungal policy for details.
– No one antibiotic should be used for greater than 10 days. The options are to stop, change and/or discuss with microbiology.

5. Granulocyte Transfusions.
Granulocyte transfusions are occasionally indicated in adults. Problems with these transfusions can include difficulty in achieving a large enough increment and worsening of pulmonary disease. However, when severe infections (e.g. invasive aspergillosis), or focal lesions (e.g. perianal sepsis), do not respond to appropriate antibiotic therapy and/or neutropenia is expected to be prolonged, granulocyte transfusions may be useful. The use of G-CSF mobilised neutrophils from relatives should be considered on an individual basis (e.g. invasive aspergillosis). **These must be irradiated**. Remember to consider any ongoing trials.

1.4.2 Specific Infections In Neutropenic Patients

Intravenous Catheter-Related Infection

- Presumed catheter related bacteraemia / fungaemia
  - Initial empirical antibiotic therapy and then modify antibiotics according to isolates.
  - Treat for at least 10 to 14 days (longer if neutropenic).
  - Remove catheter if cultures remain positive after 48 hours of therapy or if proven catheter related infection with fungi, *Staphylococcus aureus*, *Pseudomonas* spp or *Mycobacterium* spp

- Exit site infection or tunnel infection
  - Initial empirical therapy with teicoplanin.
  - Treat for at least 10 to 14 days or longer until infection resolved.
  - Modify antibiotic therapy according to isolates.
  - Remove catheter if progression or tunnel infection or if blood cultures positive for fungi, *Staphylococcus aureus*, *Pseudomonas* spp or *Mycobacterium* spp

Cytomegalovirus (CMV)
In addition to the standard treatment options for CMV discussed on page 8, Immunoglobulin IV could be given in the presence of end-organ CMV disease (especially pneumonitis) at a dose of 400 mg/kg on day 1, 4, 8, 12, and 16. Discuss with Virology.

Varicella Zoster Virus (VZV)*
Use aciclovir (10mg/kg tds) as initial IV therapy (discuss any dose reductions for renal impairment with the pharmacist). You can consider the use of valaciclovir (1g tds) when the patients condition is stable

Diarrhoea*
This should not be treated with antibiotics unless bloody diarrhoea with increased stool volume and abdominal pain are present. Then metronidazole (400mg tds) oral should be given while awaiting the results of faecal *Clostridium difficile* toxin investigations. Standard therapy is for 10 days but can be increased to a maximum of 14 days depending on symptoms. Severe C.*difficile* should be treated with metronidazole + oral vancomycin (500mg qds) - as per trust protocol (Nb vancomycin levels should be monitored, especially if renal impairment)

Clostridium difficile disease;
Mild = Stool 3-5 x/day and WCC <12
Severe = WCC >20 or Temp >38.5 or Creatinine >50% baseline or complications (e.g. surgical abdomen).

If an infectious cause for diarrhoea is not likely and both stool cultures and C. diffile toxin testing (at least one) are negative then the use of loperamide could be considered.

Enteric Virus Infection*
Rotavirus, Adenovirus, Calicivirus (small round structured viruses), Astrovirus.

Send stool sample for Electron microscopy (EM) and for viral culture (VC) in universal container.

Haemorrhagic Cystitis
Polyomavirus (BK), Adenovirus.
Send urine specimen for Electron microscopy (EM) and for PCR in universal container.

Respiratory Virus Infection*
(RSV, Influenza A/B, Parainfluenza 1,2,3, Adenovirus).

All inpatients and attending transplant outpatients with acute respiratory symptoms should be screened by NPA to screen for viral infection prior to their admission to a general haematology/ oncology ward or day assessment area.
  – The NPA should be performed by someone who has been trained by an experienced operator.
  – The initial screen will be performed by direct immunofluorescence (IFT) and by PCR.
  – All subsequent screening tests will be performed by IFT only. Once a patient is free of symptoms and has had 2 consecutive negatives by IFT, then they will be considered negative.

Inpatients
  – Haematology inpatients testing positive in the first screening test will need to be moved to a negative pressure room.
  – Return to the Haematology ward will be contigent upon absence of symptoms and 2 consecutive negative NPA by direct IFT.
  – This policy is the same as previously with the exception that the initial screening test will include PCR.

Outpatients
  – All transplant outpatients with respiratory symptoms will be encouraged to contact the BMT CNS before they attend the hospital. In outpatients known to be positive, the transplant team will discuss measures to minimize outpatient visits.
  – All transplant outpatients with respiratory symptoms (even without a positive NPA) should attend ward 4 West B where one sideroom has been designated for respiratory isolation. This room will contain only essential equipment (couch, bedside table, stethoscope). Curtains and any other non-essential equipment will be removed.
  – The Transplant team will liase with the ward 4 West B staff and ensure the room is available before a patient attends. Screening of patients by NPA will be as for inpatients.
  – All staff dealing with the patient should wear gown, masks and gloves. The appropriate 'Isolation' sign should be posted on the door.
  – After the patient has been seen and has left ward 4 West B, the member of staff dealing with the patient should perform a wipedown of horizontal surfaces (sink, couch, bedside table) with Milton disinfectant. Gowns, gloves and apron should be discarded in the room and the staff member should then wash his/her hands.

- The following day, this room will be the first to be cleaned and then other patients will be free to use this room.
- Isolation of such patient will continue until they are free of symptoms and have 2 consecutive negative NPA by direct IFT.

If Virology tests positive;

- *RSV**
  Discuss with Virology. There is no treatment with proven efficacy.
  Nebulised Ribavirin, 6g (in 100ml water for injection) per day.
  Administered as follows: Nebulise either over 12 to 16 hours or nebulise for 2 hours then stop for 6, repeat for a total of three times in a day.
  Please liaise with pharmacist about the nebuliser equipment needed for this.

  Plus

  If patient hypoxic or abnormal chest X-ray/CT, consider intravenous ribavirin.
  Discuss with virology.
  Repeat NPA at day 3.

- *Influenza**
  Start treatment with zanamivir or oseltamivir after discussion with virology.
  The zamamivir dose is 10mg inhaled twice a day. The dose of oseltamivir is 75mg bd orally. Treatment is for at least five days, but may continue for longer.
  In cases where a patient appears to have developed influenza while an inpatient, prophylactic zanamivir or oseltamivir treatment of other neutropenic or immunocompromised patients in the same ward area may be considered. This should be done in consultation with virology and infection control.

- *Parainfluenza**
  Discuss treatment with Virology.

- *Adenovirus**
  Discuss treatment with Virology. Consider cidofovir and discuss doses with Pharmacy and virology.

Staphylococcus aureus (Methicillin sensitive)
In proven line associated *S.aureus* bacteraemia intravenous treatment with flucloxacillin should be given for at least 14 days. If the heart valves are abnormal, the fever is slow to settle or no focal lesion is seen, a bone scan and echocardiogram are indicated. **Indwelling vascular devices must be removed unless there are pressing clinical or comorbidity reasons to retain it.**

Methicillin Resistant Staphyloccocus aureus (MRSA)*
In proven line-associated bacteraemia, intravenous teicoplanin should be given for at least 14 days. **Intravenous catheters must be removed.** There is a potential for dissemination and this should be investigated as for methicillin sensitive S. aureus (as above).

* Infection control procedures and protocols in place. Please see trust protocol on Freenet (clinical policies) or contact the Infection Control team (x35216)

1.4.3 Fever With Pulmonary Infiltrates

- Any drop in oxygen saturation or objective evidence of dyspnoea is an emergency. Immediate CXR and therapy must be initiated without delay. Patients with a normal CXR should proceed to a thoracic CT scan.
- At least two sets of blood cultures should be taken: one from central line and one from a peripheral vein
- If a significant pathogen is isolated, treat appropriately.
- An early bronchoscopy is important in all HSCT patients. Samples should be taken by hand by medical staff to microbiology, virology and cytology. Please discuss all bronchoscopies with micro staff to ensure all the relevant tests are done.
- Pleural effusions should be tapped early (diagnostically) where possible.
- Focal lesions should raise the possibility of fungal infection or of an abscess. CT scanning can be very helpful.
- Serum for Legionella and atypical pathogens should be collected. Urine should be sent for Legionella and pneumoccocal antigens.

PCP
– High dose co-trimoxazole for a total of 21 days: 120mg/kg/day in 2-4 divided doses intravenously for 10 days followed by the same dose orally for 11 days.
– Calcium folinate (folinic acid - 15mg po/iv once daily) should always be given with high dose co-trimoxazole.
– In proven episodes where the patient is hypoxic, give steroids AT THE START of therapy: Prednisolone 40 mg po bd for 5 days, 40 mg od for 5 days, 20 mg od for the remainder of therapy. (40mg Prednisolone is equivalent to 160mg Hydrocortisone). Remember to tail down the dose of steroids gradually.

1.4.4 Fever with pulmonary infiltrates in HSCT patients (after bone marrow recovery)   - not in respiratory failure

1.4.5 Empirical Therapy Of Acute Respiratory Failure.

Requirement of FiO_2 35% or active consideration for ventilation. Consider involving the Patient At Risk and Resuscitation Team (PARRT).
For all the following patient types also consult the antifungal guidelines for the appropriate treatment option.

Neutropenic

General
Meropenem + Azithromycin + Amikacin + Caspofungin.
(For adults with lymphoproliferative disease, ALL, or on steroids consider high dose Co-trimoxazole and CMV treatment).

HSCT patients or chemotherapy with prior Fludarabine / Cladribine

Meropenem + aminoglycoside + Azithromycin + High-Dose Co-trimoxazole + Caspofungin
Consider CMV treatment (check PCR status).
Caution: this regimen may represent a large fluid load.

Non-neutropenic

HSCT (within 6 months) or Heavy immunosuppression (e.g. long term high dose steroids
or chronic GVHD) or prior Fludarabine / Cladribine
High-Dose Co-trimoxazole + consider Azithromycin + consider CMV treatment.

See page 8/9 for notes on CMV therapy and page 16 regarding the use of steroids for PCP.

```
                    Productive
                     cough?
                                                    YES
                        │ NO
                        ▼
                 Arrange bronchoscopy                    Co-Amoxiclav
                 Treat as if at risk from CMV            (and consider
                        │                                 Azithromycin)
                        ▼
            Well                    Unwell
       (dyspnoea only)
                        │                   │
                        ▼                   ▼
          High dose            Azithromycin
          co-trimoxazole       plus high dose co-trimoxazole
                        │       and continue itraconazole
                        │       in allogeneic HSCT patients
                        ▼
          Deterioration or no                     continued deterioration
          response after 48hrs -                  follow respiratory failure
          bronchoscopy                            protocol (section 1.4.5)
```

2. THE FEBRILE NON-NEUTROPENIC PATIENT

These patients should be treated as any other patients*. Their infections and the antibiotics they need are different from those of neutropenic patients. Many of the pyrexial episodes may be due to mild viral illness for which antibiotics are not indicated.

*Except prior HSCT (within 6 months), chronic GvHD, or fludarabine / cladribine patients in whom CMV and PCP will need to be excluded.

General Notes

- All febrile patients should have 2 sets of blood cultures, and an MSU or CSU before antibiotics are given. Itraconazole trough levels should be measured if patient is on itraconazole prophylaxis. Other samples such as sputum and pus are invaluable.
- These are empirical guidelines and may need modification in the light of subsequent isolation of an organism.
- Further detail including options for Beta-lactam allergy can be found on Freenet or in the Adult Antibiotic Prescribing Aid

Candidosis
Vaginal: Fluconazole po 150mg single dose.
Oral: Amphotericin B suspension 500mg qds (+/- lozenges).
Oesophageal or failure to respond: Fluconazole po 100mg od for 7 days.

Cellulitis
Minor infections: Oral flucloxacillin.
Otherwise: Intravenous flucloxacillin and benzylpenicillin

Chest Infection
Non-severe; Amoxicillin +/- clarithromycin
Severe; Co-amoxiclav + clarithromycin
Hospital acquired pneumonia; Co-amoxiclav.

Haematuria
Send urine specimen to Virology (EM and VC) and Microbiology.

Herpes Zoster*
Cases may be admitted to designated non positive pressure side rooms if no other immunocompromised patients are on the ward. Otherwise or for patients with disseminated disease discuss with the Infectious Diseases team / ward for admission to a negative pressure side room.unless their clinical condition precludes this. Ensure that both the Virology department and Occupational Health Unit are notified. Ask Virology department to take viral scrape for DIF, PCR and EM.

For non-disseminated infection treat with Aciclovir 10mg/kg tds iv or Valaciclovir 1g tds po (if patient stable) at least until all vesicles have scabbed and the patient has been afebrile

for 48 hours (in HSCT patients this is rarely less than one week). If disseminated use iv Aciclovir 10mgs/kg 8 hourly.

For eye involvement, add Aciclovir 3% eye ointment (topically 5 times daily and continuing for at least 3 days after complete healing) and consider a formal ophthalmic assessment. Topical 5% cream is also available for facial lesions.

Outpatients who are well and have no evidence of dissemination and can reliably report progress daily by telephone, may be treated with acyclovir 800mg 5x a day or valaciclovir 1 g 3x a day for 7 days – both oral.

Intravenous Catheter-related Infection (See 1.4.2 above)
Fever in such patients should not automatically be attributed to the line. If the line is infected, coagulase negative staphylococci are the most likely organisms. It is essential that two sets of blood cultures (from separate sites) are taken before treatment. In the absence of strong clinical evidence, therapy with glycopeptides should not be initiated. If immediate treatment for a line infection with another focus is required, Teicoplanin plus therapy directed at that focus should be used.

Severe Staphylococcus aureus soft tissue infection may be treated with flucloxacillin plus oral fusidate sodium.

Meningitis*
Treatment should be discussed with the Microbiology Department in the light of results of investigations. If immediate treatment is needed for suspected meningitis, Ceftriaxone should be given (other agents may be more appropriate after LP results are available). Vancomycin does not cross the blood brain barrier and penetrates poorly into joints.

Osteomyelitis and Septic Arthritis
First line; Flucloxacillin 1-2g qds IV (+ sodium fuscidate PO if osteomyelitis)

Septic Shock
Febrile, hypotensive patients with clinical sepsis and no apparent focus of infection: Amikacin and Meropenem (which will include cover for Yersinia, Listeria and Enterococci). In patients without lines Teicoplanin is not indicated unless known to be MRSA colonised.

Sickle Crisis
Intravenous ceftriaxone to cover infections with *Haemophilus influenzae, Streptoccocus pneumoniae* and *Salmonella* species. Likely sepsis in adults should be treated with co-amoxiclav.

Superficial mycosis
After taking scrapings use clotrimazole 1% cream to affected areas bd or tds. Treat for at least 14 days after lesions have healed. In persistent or relapsing cases or for cases of onychomycosis please discuss with the Microbiology Department.

Toxoplasmosis
Pyrimethamine 100mg po twelve hourly on day 1 as a loading dose, then 25mg po tds
Sulfadiazine 3g po/iv as a loading dose, then 2g tds.

Calcium floinate 15mg po once daily.
Treatment is likely to continue for at least 6 weeks. Discuss with microbiology.

Tuberculosis*
Start patient on quadruple therapy (with Isoniazid, Rifampicin, Ethambutol and Pyrazinamide) and contact Dr Lipman's team for further advice. They will also be able to trace contacts, etc, as required. NB: TB is a notifiable disease.

Atypical Mycobacteria
Antibiotic susceptibility varies and all cases should be discussed with micro. Initial treatment usually consists of a combination of amikacin, clarithromycin and ciprofloxacin. Treatment can be modified in light of species and sensitivity results but treatment is usually up to 6 months in duration.

Urinary Tract Infection
– Uncomplicated : Nitrofurantoin (contraindicated in G6PD deficiency, in this instance use trimethoprim) po for 3-5 days in women (not in 3rd trimester of pregnancy), 5-7 days plus urological investigation in men.
– Pyelonephritis: Gentamicin iv initially, then be guided by culture results.

* Infection control procedures and protocols in place. Please see trust protocol on Freenet (clinical policies) or contact the Infection Control team (x35216)

3. GUIDELINES ON VACCINATION

(a) Haematological Malignancy and Haematopoetic Stem Cell Transplantation.

Inactivated vaccines do not represent a danger to immunocompromised persons. These vaccines comprise DTP, Inactivated Polio vaccine (Salk vaccine), Hib, Hep B, Hep A, human papillomavirus, influenza, Pneumococcal, Meningococcal C (A/C/Y/W135 quadrivalent vaccine is required if participating in the Haj or if visiting endemic areas i.e. Middle East, Africa and Asian subcontinent), as well as Cholera and inactivated Typhoid vaccine (NB: NOT the new oral vaccine). The inactivated polio vaccine is only available in combination with diptheria (low dose) and tetanus ('Revaxis').

The immune response of an immunocompromised person to the vaccine antigens may not be as good as that of a healthy individual, so that higher doses and more frequent boosters may be required.

If vaccination with an inactivated vaccine is needed in a patient about to undergo chemotherapy or radiotherapy, it should precede the treatment by at least two weeks. The antibody response may be sub-optimal if vaccination is carried out closer to, or during treatment.

Live vaccines (e.g. BCG, MMR, oral Sabin polio vaccine, Live Attenuated Typhoid, Yellow fever, Vaccinia, Chickenpox or Zoster [note that the zoster vaccine contains a higher dose of virus than does the chickenpox vaccine] vaccines) are contraindicated in this group of patients but those with leukaemia in remission who have not received chemotherapy for at least 12 months may receive live vaccines safely. Maintenance treatment for ALL in remission should be considered to be immunosuppressive chemotherapy.
Live vaccines should not be administered until 2 years after HSCT. This period may be prolonged indefinitely for patients receiving continuous immunosuppression for GVHD.

Oral polio vaccine should NOT be administered to household contacts of immunocompromised patients. Inactivated polio vaccine should be used instead. (note inactivated polio vaccine is now only available in combination with diphtheria (low dose) and tetanus).

Offer influenza vaccine to family members who will visit the HSCT patient during October-April.

If family members do not have a history of chickenpox, test them for VZV IgG and give live attenuated chickenpox vaccine if seronegative. If they develop a rash (uncommon) they should avoid contact with immunocompromised patients.

Recipients of BCG should avoid contact with immunocompromised patients for 1 month

Immunisation Programme for HSCT Patients (ALLO OR AUTO)

One Year Post HSCT

Check functional antibody titres (pneumococcal, haemophilus influenza B, tetanus) pre-vaccination. Then give;
- Revaxis (combined diphtheria [low dose], tetanus and inactivated polio vaccine) 3 doses 4 weeks apart.
- Hib vaccine (combined with meningococcal C vaccine at Royal Free – see below)
- Meningococcal C vaccine. If participating in the Haj give A/C/Y/W135 quadrivalent vaccine. As meningococcal A/C vaccine is no longer available, patients living in a country with a high prevalence of serotypes A and C may also require the quadrivalent vaccine
- Influenza vaccine.
- Hepatitis B vaccine – best results are achieved with a 0, 1month, 6 month regime
- Pneumococcal (23 valent) Vaccine. If the patient has chronic GvHD/hyposplenic state or is undergoing continuing immunosuppression, Penicillin V prophylaxis (500mg po bd) should also be given.

3 months following vaccination, re-check titres for functional antibodies. Aim for tetanus toxoid >1IU/ml, haemophilus influenza B >1mg/l and pneumococcal antibody >50mg/l.

2 Years Post HSCT

- MMR II vaccine, 2 injections 6-12 months apart. No clear evidence for benefit of second dose so this can be omitted if desired

Note MMR is contraindicated in the presence of immunosuppression or GVHD. HSC transplant recipients are presumed immunocompetant at >24 months post-transplant if they are off all immunosuppressive therapy and have no evidence of GVHD.

Every Year Thereafter

- Give Influenza vaccine to patients with chronic GVHD or who are undergoing continuing immunosuppression.
- Check Pneumococcal antibodies if the patient has chronic GVHD / hyposplenic state or is undergoing continuing immunosuppression. Re-vaccinate at 5 year intervals if the antibody level is less than 50mg/l.

(b) Vaccinations Pre-splenectomy Or In Patients With Functional Hyposplenism

One month to fourteen days before splenectomy, or as early as possible before operation or when functional hyposplenism is diagnosed:

- Give Pneumococcal vaccine. Antibody levels should be monitored every year and re-vaccination considered at five years if antibody level less than 50mg/l.
- Give Hib vaccine (single dose)

- Give Meningococcal C vaccine. Other meningococcal vaccines are only necessary if patients are travelling to an area of increased risk of infection, or those for whom it is specifically recommended i.e. contacts of cases, Haj pilgrims (see above).
- These patients also require Penicillin V prophylaxis (500mg po bd) for life. If penicillin allergic, use Erythromycin 250mg bd po
- Patients should also be given advice regarding travel to malarious areas. Please liase with a travel clinic or the ID team.

4. THERAPEUTIC DOSES OF ANTI-INFECTIVE DRUGS
AND ADMINISTRATION DETAILS

- The following are general guidelines for therapeutic doses of anti-infective drugs for haematology patients. Specific clinical situations may dictate modifications.
- For further advice about dosage modifications in renal/liver impairment or drug administration, contact the Specialist Haematology Pharmacists (Bleep 1873, 1960, 2054) or Medicines Information (Extension: 33114). Out of hours you can access the pharmacist via switchboard.
- In G6PD deficiency avoid Nitrofurantoin, Quinolones (eg ciprofloxacin), Sulphonamides (including co-trimoxazole)and Dapsone.
- For further information on reconstitution and administration of intravenous doses, refer to the 'Guidelines for Intravenous Drug Administration' and the individual products summary of product characteristics.

ACICLOVIR

Dose *Check indication, immune status, renal function and age before prescribing. Use ideal body weight.*
For VZV treatment: 10 mg/kg iv tds
                                         (or Valaciclovir 1gm tds as out patients)
For HSV treatment: 5mg/kg iv tds or 400mg po 5 times a day
For HSV prophylaxis: 400mg po tds

Dose Reduction in Renal Impairment
- For Intravenous Doses

Creatinine Clearance (ml/min)	Dose	Frequency
26 - 50	Unit dose 5-10 mg/kg	Every 12 hours
10 - 25	Unit dose 5-10 mg/kg	Every 24 hours
<10	Half unit dose 2.5-5 mg/kg	Every 24 hours

- For Oral Doses

Creatinine Clearance (ml/min)	Dose	Frequency
26 - 50	Dose as normal	Dose as normal
10 - 25	Simplex: 200mg Zoster: 800mg	3-4 times a day Every 8 – 12 hours
<10	Simplex: 200mg Zoster: 400mg – 800mg	Every 12 hours Every 12 hours

- For Valaciclovir Doses

Creatinine Clearance (ml/min)	Dose	Frequency
30-50	Zoster Dose as normal renal function	
15-30	Zoster 1g	Every 12 hours
<15	Herpes Zoster: 1g	Every 24 hours

Dose Adjustment In Liver Impairment; Dose adjustment not required.

AMIKACIN

Courses exceeding 15g in total must be discussed with Microbiology/Infectious Diseases due to very high risk of ototoxicity. Regular audiometry should be performed. Discuss need for regimen adjustment and frequency of level monitoring with Microbiology – patients with normal levels and stable renal function should have levels monitored twice weekly.

Dosing:
Standard dose is 15mg/kg amikacin IV od. Use ideal body weight (IBW)
These regimens should be used in all patients **unless the following contraindications apply:**
1. The dose is being given prophylactically (see Surgical Prophylaxis/Urinary Catheter Policies)
2. CrCl <40ml/min*
3. Age over 80y
4. Pregnancy
5. Burns
6. Ascites, severe liver disease, jaundice
7. Cystic Fibrosis

If CrCl >40 but contraindications to once daily dosing apply, give 7.5mg/kg iv bd. Take both peak and trough levels across the third dose and wait for results before giving the fourth. Keep trough < 10mg/L and peak 20-30 mg/L.

For the full guidance refer to the once daily guidelines for gentamicin and amikacin on Freenet.

Therapeutic Monitoring
Take first trough level before the 3rd dose. Await result before giving the 4th – re-dose 15mg/kg if trough level <5 mg/L Monitor trough levels (22-23 hours post-dose) twice weekly if stable renal function.

Dose Adjustment In Renal Impairment
Discuss with Microbiology and Pharmacy.
When initiating therapy the following dose alterations are necessary:
− CrCl 30-39ml/min give 15mg/kg every 36h. Trough level before 2nd dose. Await result before giving 2nd dose. Only give 2nd dose if level <5 mg/L.
− CrCl 20-29ml/min give 15mg/kg every 48h. Trough level before 2nd dose. Await result before giving 2nd dose. Only give 2nd dose if level <5 mg/L.
− CrCl <20ml/min give 7.5mg/kg stat. Trough level 22-23h later. Await result before giving next dose. Only give dose if level <5 mg/L. This process is repeated daily.

Levels must be monitored to allow determination of dose and dosage intervals.

Dose Adjustment In Liver Impairment
Dose adjustment not required, but use with caution in patients with ascites.

AMOXICILLIN

Dose
Oral: 250-500 mg tds
Intravenous: 500 -1g tds

Dose Adjustment In Renal Impairment
If CrCl >10ml/min, dose as normal.
If CrCl <10ml/min, give 250mg po/iv tds.

Dose Adjustment In Liver Impairment
Dose adjustment not required.

LIPOSOMAL AMPHOTERICIN (AMBISOME)

Refer to antifungal policy for all antifungal information.

AZTREONAM

Dose   2g iv tds

Dose Adjustment In Renal Impairment

Creatinine Clearance (ml/min)	Dose	Frequency
30 – 50	Dose as normal	Dose as normal
10 – 30	1-2g loading dose, then 50% of normal dose	Every 8 hours
<10	1-2g loading dose, then maintenance of 25% of appropriate normal dose	Every 8 hours

Dose Adjustment In Liver Impairment
Dose adjustment not required.

AZITHROMYCIN

Dose
Oral: 500mg od
Intravenous: 500g od

Duration of treatment = 3 days
For severe infections, use IV for at least 2 days followed by an oral dose to complete 7-10 days.

Dose Adjustment In Renal Impairment

Dose adjustment not required but in severe renal impairment contact pharmacist for specialist advice.

Dose Adjustment In Liver Impairment

Use in hepatic failure contraindicated – jaundice reported

BENZYLPENICILLIN

Dose   1.2g iv qds

Dose Adjustment In Renal Impairment

Creatinine Clearance (ml/min)	Dose & Frequency
20 – 50	Dose as normal
10 – 20	600mg-2.4g every 6 hours depending on severity of infection.
<10	600mg-1.2g every 6 hours depending on severity of infection.

Dose Adjustment In Liver Impairment

Dose adjustment not required, but monitor LFT's for cholestatic jaundice.

CEFOTAXIME

Dose   2g iv tds up to 12g daily, given in 3-4 divided doses, in very severe infections.

Dose Adjustment In Renal Impairment

It is only necessary to reduce the dose in severe renal impairment.
If CrCl <10ml/min: give 1g every 8-12 hours.

Dose Adjustment In Liver Impairment

Dose adjustment not required.

CEFTAZIDIME

Dose   Usually 2g iv tds

Dose Adjustment In Renal Impairment

Creatinine Clearance (ml/min)	Dose	Frequency
31 – 50	1–2g	Twelve hourly
16 – 30	1–2g	Every 24 hours
6 – 15	500mg-1g	Every 24 hours
< 5	500mg-1g	Every 48 hours

Dose Adjustment In Liver Impairment
Dose adjustment not required.

CEFTRIAXONE

Dose   1g to 2g iv every 24 hours

Dose Adjustment In Renal Impairment
If CrCl < 10ml/min, reduce the dose to 1-2g daily.

Dose Adjustment In Liver Impairment
Avoid in patients with liver disease (ceftriaxone has been associated with pseudo-lithiasis in some patients).
If the patient already has renal has a further dose reduction may be necessary

CIPROFLOXACIN

Dose   Oral: 500mg bd
                                                 Intravenous: 400mg bd

Dose Adjustment In Renal Impairment
If CrCl < 20ml/min, give 50% of normal dose (i.e. unit dose od or half unit dose bd).

Dose Adjustment In Liver Impairment
Dose adjustment not required.

Avoid in G6PD deficiency

CLARITHROMYCIN

Dose
Oral: 250mg - 500 mg twice a day;
Intravenous: 500 mg twice a day.

Dose Adjustment In Renal Impairment

Creatinine Clearance (ml/min)	Dose & Frequency
30 – 50	Dose as in normal renal function
10 – 30	IV: 250 - 500mg every 12 hours Oral: 250 - 500mg every 12 – 24 hours
<10	IV: 250mg every 12 hours Oral: 250mg every 12 – 24 hours

Dose Adjustment In Liver Impairment
Use with caution as hepatic dysfunction, including jaundice, has been reported.

Note: Potent inhibitor of cytochrome P450 enzymes

CO-AMOXICLAV (AUGMENTIN®)

Dose

Oral:	375mg - 625mg tds
Intravenous:	1.2g tds

Dose Adjustment In Renal Impairment

Creatinine Clearance (ml/min)	Dose & Frequency
30 – 50	Dose as in normal renal function
10 – 30	IV: 1.2g every 12 hours Oral: dose as normal
<10	IV: 1.2g stat followed by 600mg –1.2g every 12 hours Oral: 375mg three times a day

Dose Adjustment In Liver Impairment
Dose adjustment not required, but monitor LFT's for cholestatic jaundice.

CO-TRIMOXAZOLE

Dose

Prophylaxis:	960mg po bd on 2 consecutive days (Tuesday and Wednesday).
High dose:	120mg/kg/day in 2-4 divided doses po or iv. With high dose treatment, Folinic Acid 15mg po daily should be given.
Standard dose:	60mg/kg/day in 2-4 divided doses

Dose Adjustment In Renal Impairment
Dose adjustment not required for prophylaxis treatment.
Dose adjustment only required for patients on high dose co-trimoxazole treatment. **Levels must be monitored.**

Creatinine Clearance (ml/min)	Dose
> 30	Normal dose
15 – 30	60mg/kg for 3 days bd, then 30mg/kg/day bd
<15	Contra-indicated unless patient on haemodialysis Seek specialist advice

Dose Adjustment In Liver Impairment
Dose adjustment not necessary but co-trimoxazole can induce increases in liver enzymes.

Avoid in G6PD deficiency

ERYTHROMYCIN

Dose

Oral:	500mg qds;

Intravenous: 1g qds.

Dose Adjustment In Renal Impairment
A dose reducuction is required in severe renal impairment (due to the risk of irreversible deafness).
If CrCl >10ml/min, dose as normal
If CrCl <10ml/min, give 50-75% of normal dose (maximum 1.5g daily).

Dose Adjustment In Liver Impairment
Dose adjustment not required, but use with caution and monitor LFT's for cholestatic jaundice.

Note: Potent inhibitor of cytochrome P450 enzymes

ETHAMBUTOL

Dose
15mg/kg oral once daily. (Monitor visual acuity)

Dose Adjustment In Renal Impairment

Creatinine Clearance (ml/min)	Dose
10 - 50	7.5 - 15 mg/kg/day
<10	7.5 - 10 mg/kg /day

Dose Adjustment In Liver Impairment ; Dose adjustment not required.

FLUCLOXACILLIN

Dose   250mg - 500mg po QDS
                                      500mg-2g iv QDS
                                      Consult Microbiology for dosing guidance

Dose Adjustment In Renal Impairment
<10ml/min CrCl a maximum dose of 4g per 4 hours in divided doses can be used.

Dose Adjustment In Liver Impairment
Dose adjustment not required but monitor LFT's for cholestatic jaundice.

FLUCONAZOLE

Consult the antifungal policy for all dosing information

FOSCARNET

Dose   90mg/kg bd iv bd OR 60mg/kg iv tds
.

Dose Adjustment In Renal Impairment
The following is a guideline only. Contact the Specialist Pharmacists for more tailored dosing.
Doses can be tailored to twice daily administration. All reductions MUST be discussed with the pharmacist

Creatinine Clearance (ml/min)	Dose	Frequency
20 - 50	28mg/kg	Every 8 hours
10 - 20	15mg/kg	Every 8 hours
< 10	6mg/kg	Every 8 hours

Dose Adjustment In Liver Impairment;
Dose adjustment not required.

FUSIDATE SODIUM

Dose   Oral and intravenous: 500mg tds.
If patient < 50kg body weight :   6-7mg/kg 8 hourly, all routes.

Dose Adjustment In Renal Impairment
Dose adjustment not required.

Dose Adjustment In Liver Impairment
Consider dose adjustment in moderate to severe liver impairment.

GANCICLOVIR

Dose
For pre-emptive treatment: 5 mg/kg bd iv. G-CSF should be given concurrently.

Dose Adjustment In Renal Impairment

Creatinine Clearance (ml/min)	Dose	Frequency
> 70	5mg/kg	Twelve hourly
50 – 69	2.5mg/kg	Twelve hourly
25 – 50	2.5mg/kg	Every 24 hours
10 –25	1.25mg/kg	Every 24 hours
<10	Consult specialist advice form Pharmacy	

Dose Adjustment In Liver Impairment
Dose adjustment not required

GENTAMICIN

This must be prescribed on the approved label which is specific to Gentamicin

Dose
Once daily dosing : Initial dose 7mg/kg iv in 100ml glucose 5% over 1 hour.
Nb – use ideal body weight = [males 50kg, females 45kg] + 2.3 kg per inch over 5ft

Levels must be taken 6 -14 hours post dose. A Microbiologist must interpret the first level, before the second dose is prescribed. Levels need to be monitored regularly and dose and dosing interval modified accordingly. Refer to the once daily guidelines on Freenet. Potential dosing intervals can be predicted as follows:

Creatinine Clearance (ml/min)	Dosing Interval
>60	24 hrs
40 – 59	36 hrs
20 – 39	48 hrs

Dose In Renal Impairment
If CrCl 20 – 60, give 2mg/kg iv bd. Take both peak and trough levels at the third dose and wait for results before giving the fourth.
If CrCl < 20ml/min, give 2 mg/kg od iv. Do a trough level at 24 hours, and wait for a trough level of <2mg/litre before giving next dose.

Dose In Liver Impairment
Dose adjustment not required, but use with caution if patient has ascites.

INTRAVENOUS IMMUNOGLOBULINS

Dose
Intravenous: 400mg/kg/day. Regime varies.

Dose Adjustment In Renal Impairment
Dose adjustment not required.

Dose Adjustment In Liver Impairment
Dose adjustment not required.

ISONIAZID

Dose
Oral and intravenous: 5mg/kg once daily (10mg/kg/day for tuberculous meningitis). Usual adult dose is 300mg once daily.

Dose Adjustment In Renal Impairment
In severe renal impairment (CrCl <10ml/min), give a maximum dose of 200mg once daily.

If liver function normal, can give 300mg once daily

Dose Adjustment In Liver Impairment
Avoid in liver dysfunction as increase in idiosyncratic hepatotoxicity. Only initiate therapy if expected benefits exceed risk of further hepatic injury.

ITRACONAZOLE

Refer to antifungal policy for all antifungal information.

LENOGRASTIM GCSF

Normal dose 263mcg daily
Dose reduction not required in renal failure

MEROPENEM

Dose
1g iv tds

Dose Adjustment In Renal Impairment

Creatinine Clearance (ml/min)	Dose	Frequency
26 - 50	500mg - 2g	12 hourly (bd)
10 - 25	500mg - 1g	12 hourly (bd)
< 10	500mg - 1g	Every 24 hours (od)

Dose Adjustment In Liver Impairment
Dose adjustment not required but monitor transaminase and bilirubin.

METRONIDAZOLE

Dose
Oral: 400mg tds (max 2 weeks therapy)
Intravenous: 500mg tds

Dose Adjustment In Renal Impairment
Dose adjustment not required unless severe renal impairment.
If CrCl < 10ml/min, give normal dose every 12 hours.

Dose Adjustment In Liver Impairment
In severe liver dysfunction, there is impaired metronidazole clearance. Monitor patients closely.

NITROFURANTOIN

Dose
Acute uncomplicated infection 50mg every 6 hours
Severe recurrent infection 100mg 6 hourly

Dose Adjustment In Renal Impairment
Contraindicated in renal failure

Dose Adjustment In Liver Impairment
Dose adjustment not required.

Avoid in G6PD deficiency

PENTAMIDINE

Dose
Intravenous: For patients who are allergic or intolerant to high dose co-trimoxazole treatment: 4mg/kg once daily for at least 14 days..
Nebulised: Treatment: 600mg nebulised daily (treatment dose), after test dose of 150mg. (Rarely used).
Prophylaxis: 300mg nebulised every 4 weeks or 150mg every 2 weeks.
NB: Give inhaled terbutaline before each nebulised pentamidine dose.

Dose Adjustment In Renal Impairment
Dose adjustment not required for nebulised therapy.
For intravenous pentamidine therapy, if CrCl <10ml/min: consider a dose reduction to 4mg/kg for 7-10 days then alternate day to a total of 14 days or doses. Seek specialist advice from Microbiology and Pharmacy.

Dose Adjustment In Liver Impairment
Dose adjustment not required for nebulised therapy.
For intravenous pentamidine therapy, use with caution in patients with liver impairment, and monitor LFT's and blood glucose.

PIPERACILLIN / TAZOBACTAM (TAZOCIN®)

Dose
IV 4.5g four times a day

Dose Adjustment In Renal Impairment
If CrCl 20-35ml/min, give 4.5g tds
If CrCl <20ml/min, give 4.5g twelve hourly (bd)

Dose Adjustment In Liver Impairment
Dose adjustment not required.

PYRAZINAMIDE

Dose
Adults <50kg: 1.5g po once daily ;
Adults >50kg: 2g po once daily.

Dose Adjustment In Renal Impairment
Dose adjustment not required.

Dose adjustment In Liver Impairment
Avoid in liver dysfunction as idiosyncratic hepatotoxicity more common. Only initiate therapy if expected benefits exceed risk of further hepatic injury.

PYRIMETHAMINE

Dose
50mg -75mg po od   *or*   25mg po tds

Dose Adjustment In Renal Impairment
Dose adjustment not required.

Dose adjustment In Liver Impairment
Dose adjustment not required, but use with caution.

RIBAVIRIN

Dose
Nebulised for RSV (see below). Treatment is for 3 days. Discuss longer treatment with Virology. The need for iv ribavirin should also be discussed with Virology.
Dose Adjustment In Renal Impairment
Dose adjustment not required for nebulised treatment.
Dose adjustment required for intravenous ribavirin. Discuss with the Specialist pharmacists.

Dose Adjustment In Liver Impairment
Dose adjustment not required.

Administration Of Ribavirin Aerosol Dose

Reconstitute 6g in 100ml WFI. This is nebulised via a small particle aerosol generator (SPAG) as follows:

Nebulise for 2 hours then stop for 6 hours, then nebulise for a further 2 hours then stop for 6 hours, and then nebulise for a final 2 hours then stop.

Discard any unused preparation after 24 hours.

Ribavirin 6g in 300ml has also been nebulised continuously over 12 to 18 hours.

NB: Any member of staff who is, or could possibly be pregnant, should not enter the room whilst the nebulisation is taking place. It is safe to enter the room when nebulisation has stopped.

TEICOPLANIN

Dose

400mg iv 12 hourly for 3 doses then 400mg iv once daily.

Dose Adjustment In Renal Impairment

Creatinine Clearance (ml/min)	Dose & Frequency
20-50	Dose as usual for the first three dose, then give 100% dose every 48 hours. Or Give 50% every 24 hours
10-20	Give 100% of the dose every third day (i.e. every 72 hours). Or Give 30% of dose every 24 hours
<10	Give 100% of the dose every third day (i.e. every 72 hours). Or Give 30% of dose every 24 hours

Dose Adjustment In Liver Impairment

Dose adjustment not required.

RIFAMPICIN

Dose

Adults <50kg: 450mg po / iv once daily (if giving orally, ideally before breakfast)

Adults >50kg   600mg po / iv once daily (if giving orally, ideally before breakfast)

Use Rifinah (an oral combination of rifampicin and isoniazid) for the treatment of TB.

Dose Adjustment In Renal Impairment
<10ml/min CrCl 50-100% dose, consider a dose reduction.
Consult Microbiology and Pharmacy.

Dose Adjustment In Liver Impairment
Avoid in liver impairment, or do not exceed maximum of 8mg/kg per day. Only initiate therapy if expected benefits exceed risk of further hepatic injury.

Note: Potent inducer of cytochrome P450 enzymes

TRIMETHOPRIM

Dose   *200mg po bd for 7 days*

Dose Adjustment In Renal Impairment
Dose adjustment not required.

Dose Adjustment In Liver Impairment
Dose adjustment not required.

VALGANCICLOVR

Dose:
Pre-emptive therapy: 900mg bd until CMV PCR becomes negative on two occasions (or is less than 3000 genomes/ml; see current trial).

Dose adjustment in Renal impairment:

Creatinine Clearance (ml/min)	Treatment dose (mg)
40-59	450mg BD
25-39	450mg OD
10-24	450mg every 48hours
<10	Seek Specialist advice

Dose adjustment in liver impairment:
Dose adjustment not required

VALACICLOVIR

Dose
For treatment of chickenpox or zoster: 1g po tds

Dose Adjustment In Renal Impairment

Creatinine Clearance (ml/min)	Dose	Frequency
15-30	1g	bd
<15	1g	od

Dose Adjustment In Liver Impairment
Dose adjustment not required

VANCOMYCIN

Dose
Oral: 125mg qds for 7-10 days for antibiotic associated colitis
Intravenous: 1g bd. (NB: Infusion over at least 100 minutes)

Dose Adjustment In Renal Impairment
Dose adjustment not required for oral administration, only intravenous administration. Dose adjust in renal impairment according to blood levels. As a guide:

Creatinine Clearance (ml/min)	Dose & Frequency
20 - 50	500mg every 12 hours
10 - 20	500mg every 24 - 48 hours
< 10	500mg every 48 - 96 hours

Dose Adjustment In Liver Impairment; **Dose adjustment not required.**

Therapeutic Monitoring; Levels must be monitored regularly.
Peak levels should be 18-26mg/l, and trough levels should be 10 -15mg/l.

A.4.2.

Antifungal Strategy for Patients with Haematological Malignancies

Introduction

This collection of guidelines for prophylaxis, investigation, treatment and audit has been agreed by the Leukaemia and Lymphoma Board and forms the policy for management of all fungal infections in patients with haematological malignancy.

Prophylaxis

Patients receive prophylaxis according to risk category and this in turn influences their empirical therapy. Not all neutropenic patients require prophylaxis and some non-neutropenic ones with other significant risk factors (such as severe graft-versus-host disease) do. Risk is most easily categorised by therapeutic regimen, which allows the majority of associated risk factors to be taken account of. Patients receiving itraconazole should be considered early for the IV formulation if they are intolerant.

Prophylaxis should be continued until recovery of the neutrophil count ($>0.5 \times 10^9$/l) with no evidence of fungal infection.

For cyclophosphamide-containing regimens, prophylaxis should start on the day of HSCT transplant, at least 48 hours after the end of chemotherapy and continued until the neutrophil count is $\geq 0.5 \times 10^9$/L.

Regimen	Risk Category	Prophylaxis
Auto PBSC	Low	No
Lymphoma chemotherapy*	Low	No
Adult ALL chemotherapy**	High - Int	For vincristine containing blocks: Ambisome 3mg/kg three times per week for outpatients), followed by itraconazole at least 1 week after last vincristine dose. Otherwise use itraconazole and oral Amphotericin B
AML chemotherapy	High - Int	Itraconazole and oral Amphotericin B
Allogeneic transplantation	High	Itraconazole and oral Amphotericin B
High-dose Cytarabine (ara-C) or fludarabine regimens	High	Itraconazole and oral Amphotericin B

* HIV positive patients may require prophylaxis according to HIV risk

** avoid using itraconazole and voriconazole with vincristine containing regimens.

Vincristine cannot be given within 5 days of the last itraconazole or voriconazole dose.

Risk factors which may change risk category		
Colonised at > 1 site	High - Int	Itraconazole and oral Amphotericin B
Neutropenia > 5 weeks	High	Itraconazole and oral Amphotericin B
Corticosteroids > 1 mg/kg and neutrophils < 1 for > 1 week	High	Itraconazole and oral Amphotericin B
GVHD requiring steroids	High	Itraconazole and oral Amphotericin B

Graft-versus-host disease and graft rejection/failure
Patients with chronic graft-versus-host disease and those who become neutropenic again as a result of graft rejection/failure are at significant risk of invasive aspergillosis and should receive oral itraconazole prophylaxis. Those with significant gut involvement should have levels measured weekly.

Secondary prophylaxis
Patients with previously documented fungal infection (proven or probable invasive aspergillosis or other mould infections, or fungaemia) must receive secondary prophylaxis during subsequent neutropenia and graft-versus-host disease. This should be oral itraconazole (unless the previous infection broke through itraconazole prophylaxis or was unresponsive to itraconazole). AmBisome 3mg/kg/day three times per week may be given as an alternative. In cases where patients required voriconazole for treatment of the initial infection, this would also be a suitable secondary prophylactic agent.

Abnormal Liver Function Tests
Pre-existing abnormal liver function tests are not a contra-indication to azole prophylaxis, but patients should be monitored carefully and azoles discontinued if there is associated progressive hepatotoxicity.

Management of Neutropenic Fever - Investigations

At onset of fever:
- **Trough itraconazole level**

High-risk patients receiving oral itraconazole prophylaxis who become febrile are commenced on broad spectrum antibiotic therapy and should have itraconazole levels taken. Levels should be taken prior to the next itraconazole dose following the onset of fever. The time of the dose AND the time of the level must be recorded. Assays for itraconazole levels are performed twice weekly, therefore a result should be available at 96 hours to guide subsequent management. If patients have been intolerant of the oral preparation and are receiving IV itraconazole, the level can be assumed to be therapeutic and no assay need be performed.

At 96 hrs of fever:
- **HRCT chest**

If the fever persists despite 96 hours of broad spectrum empirical antibiotic therapy, a high resolution chest CT scan should be performed. If the HRCT is negative and trough itraconazole levels are greater than 500ng/ml or the patient is receiving IV itraconazole then additional antifungal agents are not required. If the HRCT is negative and the trough intraconazole levels are subtherapeutic, then the patient should be switched to IV itraconazole prophylaxis (200mg BD for 48hrs, then 200mg OD).
If there is a delay in performing the HRCT (beyond 120 hours), then patients should be treated with targeted therapy pending the CT. If the CT has features suggestive of fungal infection, then the patient should received targeted therapy.

<u>**Ongoing investigations:**</u>

- **Serum galactomannan levels twice weekly in high risk patients in centres where available**

High-risk patients should be screened twice weekly for serum galactomannan and the antigen should be assayed in bronchoalveolar lavage samples, when available.

Any new respiratory clinical feature should be investigated by CT scanning. All patients developing a new fever which persists despite 96 hours of broad spectrum antibiotic therapy at the end of neutropenia should have an upper abdominal CT scan (to exclude chronic disseminated candidosis).

Two sets of blood cultures should be taken from different sites (including central lines) in patients remaining febrile. An MSU (for fungal culture) should be sent prior to commencement of antifungal therapy and in symptomatic patients.

All patients with blood cultures positive for *Candida* species should have an upper abdominal HR CT scan and an ophthalmological examination of their fundi (for the detection of choroidoretinitis/endophthalmitis).

<u>Other</u>:

Low/low intermediate-risk patients should be managed on clinical or microbiological grounds. Consideration should be given to performing a chest HRCT at 96 hours of fever.

Targeted Therapy

Targeted therapy is given where evidence exists to suggest invasive fungal infection (CT, galactomannan PCR, blood culture), as outlined in the algorithm. This sets out the therapy to be given for the different categories of infection and causative organism and subsequent modification (if necessary) in the case of toxicity or failure of response. All cases should be discussed with the Microbiology Department. Completion of therapy will depend upon the causative organism, the extent of the infection and the response to therapy. It may be possible to switch to an oral azole to complete therapy, in which case the intravenous antifungal should be continued during the 24 hour loading period for the azole.

Modification of Therapy

Therapy should be modified as detailed in the algorithm. It should be noted that there is currently no evidence for combining antifungal agents in the management of invasive aspergillosis and this should not, therefore, be done.

Failure of treatment is defined as progression of clinical features (fever, respiratory function, hypotension, haemoptysis or chest pain) **after at least 7days of adequate antifungal treatment.**

Renal toxicity is defined as a creatinine clearance < 50mls/min (this can be calculated rather than directly measured).

Liver toxicity is defined as an elevation of transaminases and/or bilirubin to at least **3 times the upper limit of normal.**

Conflicts of Interest

The authors have all participated in sponsored meetings or have received honoraria, sponsorship or research support, from pharmaceutical companies supplying the antifungal agents named in these guidelines.

C C Kibbler, M Potter, V Gant, S Mackinnon, H Kershaw, N Saini
November 2003

Revised and re-issued: C C Kibbler, S Mackinnon, B Nadjm, A Prentice, S Norris
September 2005

Amendments: C C Kibbler, S Mackinnon, V Gant, K Thomson
October 2005

Revised and re-issued: C C Kibbler, A.Prentice, V Gant, K Thomson
February 2007

ANTIFUNGAL THERAPY FOR HIGH INTERMEDIATE/HIGH RISK NEUTROPENIC PATIENTS

When patient commenced on empirical antibacterial therapy take trough itraconazole level if on PO

↓

Fever >38C after 96 hours broad spectrum antibiotics no focus of infection

↓

Perform HR CT scan chest if not already done

↓

Significant CT lesions or other evidence of IFI?

— No → **Itraconazole level >500ng/L ?**

- No → IV itraconazole
- Yes → Continue PO itraconazole

↓

New Clinical or radiological features suggest fungal infection?

- No → **CONTINUE**
- yes → (back to Category of infection)

— Yes → **Category of infection? Discuss with Microbiology**

- Presumed aspergillus or invasive candidiasis → **CASPOFUNGIN**
- Other IFI, including zygomycosis → **Treat according to Microbiology advice**

CASPOFUNGIN → **Liver toxicity/ failure to respond**

- Yes → **AmBisome 3mg/kg/day**

Antifungal Agents: Doses

LIPOSOMAL AMPHOTERICIN (AMBISOME)

Dose in Adults & Children
Intravenous: 3mg/kg three times per week for prophylaxis (see text and algorithm)
3mg/kg/day for treatment
Give a test dose of 1mg on the first day of treatment.
N.B. Liposomal amphotericin must be infused in glucose 5% with a pre and post flush of glucose 5%.

Dose Adjustment in Renal Impairment
Monitor renal function closely. If renal toxicity supervenes swap to caspofungin or voriconazole, taking the potential for drug interactions into consideration
Dose Adjustment in Liver Impairment
Dose adjustment not required.

ORAL AMPHOTERICIN B

Dose in adults
500mg four times a day, total of 2grams.
Tablets: 5 x 100mg four times a day
or   Suspension: 5ml four times a day
Dose in children
Dose is dependant on age and weight, consult Paediatric Pharmacist for specialist advice.

CASPOFUNGIN

Dose in Adults
Intravenous: Loading dose of 70mg, then 50mg od thereafter.
NB: If patient weight >80kg: loading dose of 70mg, then 70mg od thereafter.
N.B. Caspofungin must have a pre and post flush with sodium chloride 0.9% and be infused in sodium chloride 0.9%
Dose in Children
Clinical experience limited. Seek specialist advice.

Dose Adjustment in Renal Impairment
Dose adjustment not required.

Dose Adjustment in Liver Impairment
For mild hepatic insufficiency (Child-Pugh score 5 - 6): no dose adjustment required.
For moderate hepatic insufficiency (Child-Pugh score 7 - 9): loading dose of 70mg, then 35mg od thereafter.
For severe hepatic insufficiency (Child-Pugh score > 9): there is no clinical experience but a higher exposure than in moderate hepatic impairment is expected, so use with caution in these patients.

FLUCONAZOLE

Dose in Adults
Oral: 100mg od for prophylaxis.
Otherwise 400mg OD for systemic candidosis or cryptococcosis

Intravenous: Loading dose of 800mg, then 400mg od.
Dose in Children
Mucosal candidiasis: iv/oral: 3mg/kg daily
Systemic candidiasis: iv/oral: 6-12mg/kg daily

Dose Adjustment in Renal Impairment
For adults: If CrCl <10ml/min, give a maximum daily adult dose of 150mg.
For children:
CrCl   11-40ml/min/1.73m^2
                        Dosage interval: 48 hours (or half the normal daily dose)
CrCl   <10ml/min/1.73m^2
                        Dosage interval: 72 hours (or third of the normal daily dose)

N.B. If patient established on effective haemofiltration specialist advice should be sought.

Dose Adjustment in Liver Impairment
Dose adjustment not required.

ITRACONAZOLE

Dose in Adults
  Oral solution: 200mg bd (if tolerance is a problem od dosing may be appropriate). If still intolerant, give itraconazole IV.
        When switching therapy from IV antifungal therapy load with 400mg bd for 24 hours.
Intravenous: 200mg bd for 48 hours then 200mg od

Dose In Children
Oral: 2.5mg/kg bd (max 200mg bd)
Intravenous: 2.5mg/kg od (max 200mg od)

Dose Adjustment in Renal Impairment
Dose adjustment not required.
If CrCl < 30ml/min, intravenous itraconazole should not be used as accumulation of the intravenous vehicle (cyclodextran) occurs and can result in potential toxicity.

Dose Adjustment in Liver Impairment
Itraconazole is predominantly metabolised in the liver. Therefore, in moderate to severe liver dysfunction (Child-Pugh score > 7), consider dose adjustment and only initiate therapy if expected benefits exceed risk of further hepatic injury.

VORICONAZOLE

Voriconazole can cause serious visual disturbances, these affect colour, focus and concentration. These are reversible on discontinuation of the drug.

Dose in Adults
Oral: Patients >40kg: 400mg bd for first 24 hours, then 200mg bd thereafter
             Patients <40kg: 200mg bd for first 24 hours, then 100mg bd thereafter

Intravenous: 6mg/kg every 12 hours for first 24 hours, then 4mg/kg bd

Dose in Children
In children aged 2 to 12 years:
Oral: 200mg BD
Intravenous: 7mg/kg every 12 hours

In children 12-16 years: dose as adults

Dose Adjustment in Renal Impairment
Dose adjustment is not required, for the oral formulation.
If CrCl <50ml/min, accumulation of the intravenous vehicle (SBECD) occurs, and the oral route should be used, unless the benefits outweighs the risks of intravenous treatment.

Dose Adjustment in Liver Impairment
No dose adjustment necessary in patients with acute hepatic injury, manifested by elevated liver function tests, but continued monitoring is required.
In patients with mild to moderate hepatic cirrhosis (Child-Pugh A and B, or score 5 - 9), the standard loading dose should be used, but the maintenance dose should be halved.
Voriconazole has not been studied in severe chronic cirrhosis (Child-Pugh C, or score >10).

Appendix-5

Materials

A.5.1.   <u>Equipments:</u>

Capillary electrophoresis analyzer   (Applied Biosystems 31130;California;USA)

50ml falcon tubes   (Greiner;Bio-one;North America)

Gel Doc 2000   (BioRad;California, USA)

G-Storm thermal cycler   (Biotage;Virginia;USA)

Microcentrifuge   (MSE;London;UK)

Microcentrifuge (0.25, 0.5 & 1.5 ml) tubes

                                                               (BioSc;Utah;USA)

Micropipettes tips (1-20µl and 20-200µl)

                                                               (Starlab ;USA)

Nanodrop-ND1000   (Labtech;UK)

PCR (polymerase chain reaction) machine / thermal cyclerThermal seal
                                                               (WebScientific;Cheshire;UK)

PCR microplate (PCR-96-AB-C)   (Axygen Scientific;California;USA)

PCR plate (96 well thin wall)   (Webscientfic;Cheshire;UK)

Pipetman (20 & 200-µl)   (Gilson;France)

PyroMark Q24 (Pyrosequencing)   (Biotage;Virginia;USA)

Tetrad & Dyad blocks   (MJ Research;Aberdeen;UK)

Vortex mixer   (Scientific ind;Bohemia NY;USA)

14ml yellow top (centrifuge) tubes   (TPP;Switzerland)

A.5.2. <u>Reagents:</u>

A.5.2.1.   DNA extraction & Red cell lysis

RBC lysis solution   (Gendera Puregene;Wales;UK)

Cell lysis solution   (Gentra Puregene;Wales;UK)

DNA hydration solution   (Gentra Puregene;Wales;UK)

70% Ethanol

Isopropanol

PBS (Phosphate buffered saline)   (Oxoid;Basingstoke;UK)

Protein precipitant solution for DNA (Gentra Puregene;Wales;UK)

RBC lysis solution   (Gendera Puregene;Wales;UK)

A.5.2.2.   Electrophoresis reagents:

Agrose multiple-purpose   (Bioline ltd;London;UK)

DNA Lader Low 1000bp   (Bioline ltd;London;UK)

Syber safe DNA gel stain   (Invitrogen;Oregon;USA)

TBE x10 buffer solution   (Flowgen;Nottingham;UK)

Tracing dye   (Bioline ltd;London;UK)

A.5.2.3.   Genescanning reagents:

- Gene Scan^{TM}-500 LIZ^{TM} Size standard

                         (AB applied Biosystems;California;USA)

-Hi-dye formamide   (AB applied Biosystems;California;USA)

A.5.2.4.   **Pyrosequencing reagents:**

Annealing buffer   (Biotage;Virginia;USA)

Binding buffer   (Biotage;Virginia;USA)

SNP primer S1

Streptavidin sepherose   (**GE** healthcare;Sweden)

A.5.2.5.   **PCR reagents:**

BioTaq™ DNA polymerase   (Bioline ltd;London;UK)

100mM dNTP set (Deoxynucleotide Triphosphates) dNTPs

        dATP, dGTP, dCTP, dTTP   (invitrogen;CA;USA)

                                        (Bioline ltd;London;UK)

Magnesium chloride   (Bioline ltd;London;UK)

10x polymerase buffer   (Bioline ltd;London;UK)

Sterile water (dd H_2O)

A.5.3.   <u>Primers:</u>

A.5.3.1.   **Chitotriosidase primers for genotyping:**

Oligonucletoid primers   (Sigma Genoys;Texas;USA)

Primer Name	Primer sequence (5'-3')
Chito-F'	AGCTATCTGAAGCAGAAG
Chito-R'	GGACAAGCCGGCAAAGTC

A.5.3.2. **NOD2 primers for genescanning & sequencing:**

Oligonucletoid primers   (vh bio;Edinburgh;UK)

Primer Name	Primer sequence
Exon 4 WT	5' FAM CAT CTG AGA AGG CCC TGC TCC
Exon 4 SNP8	5' HEX CAT CTG AGA AGG CCC TGC TCT-3'
Exon 4 Common Antisense	5'-CTG CCC AAC ATT CAG GCC AC-3'

Exon 8 WT	5' FAM TCG TCA CCC ACT CTG TTG CC-3'
Exon 8 SNP12	5' HEX TCG TCA CCC ACT CTG TTG CG-3'
Exon 8 Common Sense	5'-GAT GGA GGC AGG TCC ACT TTG C-3'

Exon 11 WT	5' FAM ATG GCT TCA TTC CTT TCA AGG GC
Exon 11 SNP13	5'HEX ATG GCT TCA TTC CTT TCA AGG GG
Exon 11 Common Sense	5'TGG TAC TGA GCC TTT GTT GAT GAG CTC

A.5.3.3. **NOD2 primers for pyrosequencing:**

Oligonucletoid primers   (vh bio;Edinburgh;UK)

Exon 4:	F1	5'-Biotin-GGG GCC TGC TGG CTG AGT-3'
	R1	5'-GGA AGT GCT TGC GGA GGC T-3'
Exon 8:	F1	5'-GCA GAG GGA GGA GGA CTG TT-3'
	R1	5'-Biotin-CCC TCG TCA CCC ACT CTG TT-3'
Exon 11:	F1	5'-Biotin-ACC AGA ACC AGG ATG A-3'
	R1	5'-AAA CTG AGG TTC GGA GAG CTA A-3'

A.5.4. Solutions:

A.5.4.1. Agrose gel (1.5%):

Agrose powder (0.75g) was mixed with TBEx5 (50ml) and heated in the microwave for one minute, then ethidium promide, or sybersafe (2.5µl) was added to the mixture and stirred. The mixture was poured in the electopherosis plate and was left for 15-20 minutes in order for the gel to form.

A.5.4.2. dNTP stock:

2mM dNTPs (Invitrogen) was prepared by mixing equal volumes of each dNTP (dATP, dGTP, dCTP, dTTP) to give a concentration of 25mM. This was diluted 1:12.5 in ddH$_2$O (10µl dNTP mix + 115µl ddH$_2$O) to give a final 2mM working solution, and stored at -20ºC.

A.5.4.3. Ethidium bromide:

Ethidium bromide used for staining DNA in gels was prepared as a stock solution of 10mg/ml in ddH$_2$O. It was stored at room temperature in a bottle wrapped in an aluminium foil.

A.5.4.4. MgCL$_2$ stock:

Magnesium chloride (BioLine) 50mM was diluted at proportion of 15:35 in ddH$_2$O to give a final concentration 15mM of working solution, and stored at -20ºC.

A.5.4.5. PBS buffer x1:

10 PBS tablet (Oxoid) was dissolved in a liter of ddH$_2$O to make a final 1xPBS.

A.5.4.6. TBE x10:

10xTBE (Flowgen) was diluted with ddH$_2$O to 0.5 stored at room temperature.

A.5.4.7. Tracking dye:

The tracking dye was prepared by dissolving 0.25% Bromophenol blue, 0.25% Xylene Cyanol FF, 15% Ficoll (Type 400; Pharmacia) in water and stored at room temperature. It was used to load DNA in the wells of agros gel.

Appendix-6

CHITOTRIOSIDASE as genetic determinant of sepsis in

haematological malignancy

Table A.6.1. Total febrile events in each genotype. Porportions of patients (Pts) out of each chitotriosidase genotype with febrile events whilst neutropenic (FNE) or non-neutropenic (FNnE). (P value: 1st column wild type:hete:homo, 3rd column wild type :mutant, 4th column wild type:hete, 5th wild type:homo). P value calculation by Chi square, figures $\leq$ 10 Fisher exact was used.

Total	Wild type	Mutant	Heterozygote	Homozygote
Total pt 204 (%)	122 (59.80%)	82 (40.20%)	71 (34.80%)	11 (5.39%)
Total Febrile events 317 (%; total events/genotype event)	200 (63.09)	117 (36.91)	101 (31.86)	16 (5.05)
Total 203 FNE (%;genotype total events/genotype events)	127 (62.56%)	76 (37.44%)	63 (31.04%)	13 (6.40%)
Total 114 FNnE (% genotype total events/genotype events)	73 (64.04%)	41 (35.96%)	38 (33.33%)	3 (2.63%)
Pts with FNE (% pts of FNE/ genotype pts) P = 0.399	74 (60.66%)	42 (51.22%) Wt:Mut 0.182	36 (50.70%) Wt:Het 0.178	6 (54.55%) Wt:Hom 0.484
Pts with FNnE (% pts of FNnE/ genotype pts) P = 0.754	47 (38.52%)	29 (35.37%) Wt:Mut 0.642	26 (36.62%) Wt:Het 0.792	3 (27.27%) Wt:Hom 0.205
Pts with either febrile events P = 0.413	56 (45.90%)	37 (45.12%) Wt:Mut 0.913	30 (64.79%) Wt:Het 0.623	7 (63.64%) Wt:Hom 0.208
Pts with both febrile events. P = 0.370	33 (27.05%)	17 (20.73%) Wt:Mut 0.304	16 (22.54%) Wt:Het 0.487	1 (9.09%) Wt:Hom 0.140

Table A.6.2. Non-neutropenic events in relation to chemotherapy in different chitotriosidase genotypes. Percentage calculation: FNnE in chemo relation x 100 / total FNnE in each genotype.

Febrile non neutropenic events=114	Wild type=73	Heterozygote=38	Homozygote=3
Chemo related Mean; FNnE/pt %	71 0.58 97.26	38 0.54 100	3 0.27 100
Chemo unrelated Mean; FNnE/pt %	2 0.02 2.74	0	0

Table A.6.3. Chemo-related FNnE in relation to chemo-induced neutropenia in the chitotriosidase genotypes.

FNnE=114	Wild type=73	Heterozygote=38	Homozygote=3
Pre-neutropenia (%) Mean; FNnE/pt	31 (42.47%) 0.25	18 (47.37%) 0.25	2 (66.67%) 0.18
Post-neutropenia (%) Mean; FNnE/pt	24 (32.88%) 0.20	10 (26.32%) 0.14	1 (33.33%) 0.09
Unrelated to EoN (%) Mean; FNnE/pt	16 (21.92%) 0.13	10 (26.32%) 0.14	0

Table A.6.4. Febrile events with positive bacterial cultures. (Neutopenic and/or non-neutropenic) in different chitotriosidase genotypes. (P value: 1st column wild type:hete:homo, 3rd column wild type :mutant, 4th column wild type:hete, 5th wild type:homo). P value calculation by Chi square, figures $\leq$ 10 Fisher exact was used.

Fever and positive bacterial cultures	Wild type Total=122	Mutant Total=82	Heterozygote Total=71	Homozygote Total =11
Pts with only neutropenic events. (%). Pvalue 0.564	38 (31.15%)	20 (24.39%) 0.294	18 (25.35%) 0.392	2 (18.18%) 0.199
Pts with only non-neutropenic events (%). P value 0.662	22 (10.66%)	10 (12.20%) 0.733	8 (11.27%) 0.078	2 (18.18%) 0.312
Pts with total events either neutropenia or non-neutropenia (%). P value 0.779	60 (49.18%)	38 (46.34%) 0.691	34 (47.89%) 0.862	4 (36.36%) 0.182
Pts with events, both whilst neutropenia and non-neutropenia (%). P value 0.503	9 (7.38%)	8 (9.76%) 0.547	8 (11.27%) 0.358	0 0.448

Table A.6.5. Febrile events with proven or probable IFD whilst neutopenic or non-neutropenic in different chitotriosidase genotypes. (P value: 1st column wild type:hete:homo, 3rd column wild type :mutant, 4th column wild type:hete, 5th wild type:homo). P value calculation by Chi square, figures $\leq$ 10 Fisher exact was used.

Patients with fever and Fungal positive event	Wild type; Total pts=122	Mutant; Total=82	Heterozygote ; Total pts=71	Homozygote ; Total Pts=11
Pts with only neutropenic events. (%). P value 0.582	2 (1.64)	0 Wt:Mut 0.356	0 Wt:Het 0.398	0 Wt: Hom 0.841
Pts with only non-neutropenic events. (%) P value 0.618	3 (2.46)	0 Wt:Mut 0.212	0 Wt:Het 0.250	0 Wt: Hom 0.770
Pts with total events either neutropenia or non-neutropenia (%). P value 0.283	5 (4.10)	0 Wt:Mut 0.074	0 Wt:Het 0.098	0 Wt:Hom 0.654
Pts with events, both whilst neutropenia and non-neutropenia (%)	0	0	0	0

Table A.6.6. Proportion of patients out of each chitotriosidase genotype. Events of fever (with only possible IFD) whilst neutopenic or non-neutropenic. %; Pts with event x100 / total genotype. (P value: 1st column wild type:hete:homo, 3rd column wild type :mutant, 4th column wild type:hete, 5th wild type:homo). P value calculation by Chi square, figures $\leq$ 10 Fisher exact was used.

Fungal positive event Possible IFD	Wild type; Total pts=122	Mutant; Total=82	Hetero; Total pts=71	Homo; Total Pts=11
Pts with FNE % P value 0.662	4 3.28	1 1.22 Wt:Mut 0.257	1 1.41 Wt:Het 0.249	0 Wt: Hom 0.645
Pts with FNnE % P value 0.713	1 0.82	0 Wt:Mut 0.598	0 Wt:Het 0.632	0 Wt: Hom 0.917
Pts with any febrile event % P value 0.475	5 4.10	1 1.22 Wt:Mut 0.183	1 1.41 Wt:Het 0.222	0 Wt: Hom 0.645
Pt with both febrile events %	0	0	0	0

Table A.6.7. Febrile events due to viral pathogens whilst neutopenic or non-neutropenic in different chitotriosidase genotypes. %; Pts with event x100 / total genotype. (P value: 1st column wild type:hete:homo, 3rd column wild type :mutant, 4th column wild type:hete, 5th wild type:homo). P value calculation by Chi square, figures $\leq$ 10 Fisher exact was used.

Patients with fever and viral positive event	Wild type; Total =122	Mutant; Total=82	Heterozygote Total=71	Homozygote; Total=11
Pts with only neutropenic events. (%) P value 0.624	1 (0.82)	2 (2.44) Wt:Mut 0.354	2 (2.82) Wt:Het 0.305	0 Wt:Hom 0.917
Pts with only non-neutropenic events. (%) P value 1.000	1 (0.82)	0 Wt:Mut 0.598	0 Wt:Het 0.635	0 Wt:Hom 0.917
Pts with total events either neutropenia or non-neutropenia. (%) P value 0.701	2 (1.64)	2 (2.44) Wt:Mut 0.350	2 (2.82) Wt:Het 0.327	0 Wt:Hom 0.841
Pts with fever, both whilst neutropenia & non-neutropenia	0	0	0	0

Table A.6.8. Febrile events without identifiable organism whilst neutopenic or non-neutropenic in different chitotriosidase genotypes. Culture –ve: No organism grown in a febrile patient. %; Pts with event x100 / total genotype. (P value: 1st column wild type:hete:homo, 3rd column wild type :mutant, 4th column wild type:hete, 5th wild type:homo). P value calculation by Chi square, figures $\leq$ 10 Fisher exact was used.

Fever and culture -ve event	Wild type; Total =122	Mutant Total=82	Heterozygote Total pts=71	Homozygote Total =11
Pts with only neutropenic events. (%) P value 0.068	27 (23.13%)	14 (17.07%) Wt:Mut 0.377	10 (14.08%) Wt:Het 0.171	4 (36.36%) Wt:Hom 0.154
Pts with only non-neutropenic events. (%). P value 0.243	18 (14.75%)	10 (12.20%) Wt:Mut 0.634	9 (12.68%) Wt:Het 0.720	1 (9.90%) Wt:Hom 0.343
Pts with total events either neutropenia or non-neutropenia. (%) P value 0.058	59 (48.36%)	27 (32.93%) Wt:Mut 0.029	22 (30.99%) Wt:Het 0.018	5 (45.45%) Wt:Hom 0.242
Pts with fever, both whilst neutropenia & non-neutropenia. (%). P value 0.214	13 (10.66%)	3 (3.66%) Wt:Mut 0.041	3 (4.23%) Wt:Het 0.067	0 0 Wt:Hom 0.308

Table A.6.9. Proportion of episodes of neutropenia in which patients developed bacterial FNE, probable or proven IFD, possible fungal infection, viral infection or culture negative fever in all ChT genotypes. % calculated by Febrile EoN with type of cultures / Total EoN in each genotype. (P value: 1st column wild type:hete:homo, 3rd column wild type :mutant, 4th column wild type:hete, 5th wild type:homo). P value calculation by Chi square, figures $\leq$ 10 Fisher exact was used.

Episodes of neutropenia with febrile events.	Wild type; Total EoN=282	Mutant; Total EoN=171	Heterozygote Total EoN=135	Homozygote; Total EoN=36
Number of EoN with bacterial FNE. (%) P value 0.010	69 (24.47%)	40 (23.39%) Wt:Mut 0.795	38 (28.15%) Wt:het 0.421	2 (5.56%) Wt:hom 0.004
Number of EoN with probable/ proven fungal FNE. (%). P value 1.000	2 (0.71%)	0 Wt:Mut 0.387	0 Wt:het 0.457	0 Wt:hom 0.786
Number of EoN with viral FNE. (%). P value 0.412	1 (0.35%)	2 (1.17%) Wt:Mut 0.320	2 (1.46%) Wt:het 0.246	0 Wt:hom 0.887
Number of EoN with culture negative FNE. (%). P value 0.190	49 (17.38%)	30 (17.54%) Wt:Mut 0.964	20 (14.81%) Wt:het 0.510	10 (27.78%) Wt:hom 0.131

Table A.6.10. Total febrile events regardless neutropenia in different chitotriosidase genotypes, and the type of identifiable organism if present in each febrile event associated with neutropenia. (P value: 1st column wild type:hete:homo, 3rd column wild type :mutant, 4th column wild type:hete, 5th wild type:homo). P value by Chi square, figures $\leq$ 10 Fisher exact was used.

Total febrile events=317	Wild type; Total events=199	Mutant; Total events =117	Heterozygote Total events=101	Homozygote; Total events=16
Total bact +ve events Bacterial +ve/febrile event % P value 0.031	101 50.75%	68 58.12% Wt:Mut 0.205	63 62.38% Wt:Het 0.037	5 31.25% Wt:Hom 0.069
Total fung +ve events Fungal+ve/febrile event % P value 0.705	5 2.51	0 Wt:Mut 0.097	0 Wt:Het 0.126	0 Wt:Hom 0.677
Total viral +ve events Viral +ve/febrile event % P value 0.214	2 1.01	2 1.71 Wt:Mut 0.328	2 1.98 Wt:Het 0.301	0 Wt:Hom 0.856
Total culture -ve events Culture –ve / febrile event %P value 0.029	91 45.73	47 40.17 Wt:Mut 0.336	36 35.64 Wt:Het 0.095	11 68.75 Wt:Hom 0.076

Table A.6.11. Total febrile events associated with neutropenia in different chitotriosidase genotypes, and the type of identifiable organism if present in each febrile event associated with neutropenia. (P value: 1st column wild type:hete:homo, 3rd column wild type :mutant, 4th column wild type:hete, 5th wild type:homo). P value calculation by Chi square, figures $\leq$ 10 Fisher exact was used.

Total febrile neutropenic events	Wild type; FNE=126	Mutant; FNE=76	Heterozygote; FNE=63	Homozygote; FNE=13
Number of Bact FNE Bacterial rate/FNE % P value 0.015	74 58.73%	45 59.21% Wt:Mut 0.946	42 67% Wt:Het 0.291	3 23% Wt:Hom 0.012
Number of fung FNE Fungal rate / FNE% P value 0.609	2 1.6	0 Wt:Mut 0.388	0 Wt:Het 0.443	0 Wt:Hom 0.821
Number of viral FNE Viral +ve / FNE% P value 0.393	1 0.8	2 2.63% Wt:Mut 0.317	2 3.2 Wt:Het 0.258	0 Wt:Hom 0.906
Number of culture -ve FNE Culture -ve rate / FNE% P value 0.007	49 38.89%	29 38.16% Wt:Mut 0.918	19 30.16% Wt:Het 0.238	10 77% Wt:Hom 0.008

Table A.6.12. Total febrile non-neutropenic events only in different chitotriosidase genotypes, and the type of identifiable organism if present in each febrile event associated with neutropenia. (P value:1st column wild type:hete:homo, 3rd column wild type :mutant, 4th column wild type:hete, 5th wild type:homo). P value by Chi square, figures $\leq$ 10 Fisher exact was used.

Total febrile non-neutropenic events	Wild type FNnE=73	Mutant FNnE=41	HeterozygoteF NnE=38	Homozygote FNnE=3
Number of bact FNnE Bacterial rate/FNnE% P value 0.144	27 37%	23 56 Wt:Mut 0.048	21 55 Wt:Het 0.065	2 67 Wt: Hom 0.323
Number of fung FNnE Fungal rate / FNnE% P value 0.585	3 41	0 Wt:Mut 0.259	0 Wt:Het 0.280	0 Wt:Hom 0.885
Number of viral FNnE Viral +ve / FNnE% P value 1.000	1 1	0 Wt:Mut 0.640	0 Wt:Het 0.658	0 Wt:Hom 0.961
Number of culture negative FNnE Culture -ve rate / FNnE% P value 0.392	43 59	18 44 Wt:Mut 0.162	17 45 Wt:Het 0.200	1 33 Wt:Hom 0.323

Reference List

1. Malaguarnera L. Chitotriosidase: the yin and yang. Cell Mol Life Sci 2006;63:3018-3029.

2. Ahmed N, El-Mahallawy HA, Ahmed IA, Nassif S, El-Beshlawy A, El-Haddad A. Early hospital discharge versus continued hospitalization in febrile pediatric cancer patients with prolonged neutropenia: A randomized, prospective study. Pediatr Blood Cancer 2007;49:786-792.

3. Buchheidt D, Bohme A, Cornely OA, Fatkenheuer G, Fuhr HG, Heussel G, Junghanss C, Karthaus M, Kellner O, Kern WV, Schiel X, Sezer O, Sudhoff T, Szelenyi H. Diagnosis and treatment of documented infections in neutropenic patients--recommendations of the Infectious Diseases Working Party (AGIHO) of the German Society of Hematology and Oncology (DGHO). Ann Hematol 2003;82 Suppl 2:S127-S132.

4. Ogura Y, Bonen DK, Inohara N, Nicolae DL, Chen FF, Ramos R, Britton H, Moran T, Karaliuskas R, Duerr RH, Achkar JP, Brant SR, Bayless TM, Kirschner BS, Hanauer SB, Nunez G, Cho JH. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. Nature 2001;411:603-606.

5. Hampe J, Cuthbert A, Croucher PJ, Mirza MM, Mascheretti S, Fisher S, Frenzel H, King K, Hasselmeyer A, MacPherson AJ, Bridger S, van DS, Forbes A, Nikolaus S, Lennard-Jones JE, Foelsch UR, Krawczak M, Lewis C, Schreiber S, Mathew CG. Association between insertion mutation in NOD2 gene and Crohn's disease in German and British populations. Lancet 2001;357:1925-1928.

6. Labadaridis I, Dimitriou E, Theodorakis M, Kafalidis G, Velegraki A, Michelakakis H. Chitotriosidase in neonates with fungal and bacterial infections. Arch Dis Child Fetal Neonatal Ed 2005;90:F531-F532.

7. van EM, van Roomen CP, Renkema GH, Bussink AP, Andrews L, Blommaart EF, Sugar A, Verhoeven AJ, Boot RG, Aerts JM. Characterization of human phagocyte-derived chitotriosidase, a component of innate immunity. Int Immunol 2005;17:1505-1512.

8. Holler E, Rogler G, Herfarth H, Brenmoehl J, Wild PJ, Hahn J, Eissner G, Scholmerich J, Andreesen R. Both donor and recipient NOD2/CARD15 mutations associate with transplant-related mortality and GvHD following allogeneic stem cell transplantation. Blood 2004;104:889-894.

9. Mayor NP, Shaw BE, Hughes DA, Maldonado-Torres H, Madrigal JA, Keshav S, Marsh SG. Single nucleotide polymorphisms in the NOD2/CARD15 gene are associated with an increased risk of relapse and death for patients with acute leukemia after hematopoietic stem-cell transplantation with unrelated donors. J Clin Oncol 2007;25:4262-4269.

10. Sairafi D, Uzunel M, Remberger M, Ringden O, Mattsson J. No impact of NOD2/CARD15 on outcome after SCT. Bone Marrow Transplant 2008.

11. Warner JK, Wang JC, Hope KJ, Jin L, Dick JE. Concepts of human leukemic development. Oncogene 2004;23:7164-7177.

12. Warner JK, Wang JC, Takenaka K, Doulatov S, McKenzie JL, Harrington L, Dick JE. Direct evidence for cooperating genetic events in the leukemic transformation of normal human hematopoietic cells. Leukemia 2005;19:1794-1805.

13. Jaffe ES, Harris NL, Stein H, Vardiman JW. Pathalogy & Genetics Tumours of Haematopoietic Lymphoid Tissues. Lyon: IARCPress, 2001.

14. Aplan PD. Causes of oncogenic chromosomal translocation. Trends Genet 2006;22:46-55.

15. Good RA. Relations between immunity and malignancy. Proc Natl Acad Sci U S A 1972;69:1026-1032.

16. Swerdlow AJ, Schoemaker MJ, Higgins CD, Wright AF, Jacobs PA. Cancer incidence and mortality in men with Klinefelter syndrome: a cohort study. J Natl Cancer Inst 2005;97:1204-1210.

17. Shannon K. Genetic predispositions and childhood cancer. Environ Health Perspect 1998;106 Suppl 3:801-806.

18. Shen JJ, Williams BJ, Zipursky A, Doyle J, Sherman SL, Jacobs PA, Shugar AL, Soukup SW, Hassold TJ. Cytogenetic and molecular studies of Down syndrome individuals with leukemia. Am J Hum Genet 1995;56:915-925.

19. West J, Logan RF, Smith CJ, Hubbard RB, Card TR. Malignancy and mortality in people with coeliac disease: population based cohort study. BMJ 2004;329:716-719.

20. Negri E, Talamini R, Montella M, Dal ML, Crispo A, Spina M, La VC, Franceschi S. Family history of hemolymphopoietic and other cancers and risk of non-Hodgkin's lymphoma. Cancer Epidemiol Biomarkers Prev 2006;15:245-250.

21. Chang ET, Smedby KE, Hjalgrim H, Porwit-MacDonald A, Roos G, Glimelius B, Adami HO. Family history of hematopoietic malignancy and risk of lymphoma. J Natl Cancer Inst 2005;97:1466-1474.

22. Chiu BC, Weisenburger DD, Cantor KP, Zahm SH, Holmes F, Burmeister LF, Blair A. Alcohol consumption, family history of hematolymphoproliferative cancer, and the risk of non-Hodgkin's lymphoma in men. Ann Epidemiol 2002;12:309-315.

23. Zhu K, Levine RS, Brann EA, Gu Y, Caplan LS, Hall I, Baum MK. Risk factors for non-Hodgkin's lymphoma according to family history of haematolymphoproliferative malignancies. Int J Epidemiol 2001;30:818-824.

24. Chang ET, Smedby KE, Zhang SM, Hjalgrim H, Melbye M, Ost A, Glimelius B, Wolk A, Adami HO. Dietary factors and risk of non-hodgkin lymphoma in men and women. Cancer Epidemiol Biomarkers Prev 2005;14:512-520.

25. Nishizono-Maher A, Sakamaki H, Mizukami H, Kuraki T, Minakawa K, Masuda Y. Leukaemia linked to eating disorders. BMJ 1993;306:830-831.

26. Schull WJ. The somatic effects of exposure to atomic radiation: the Japanese experience, 1947-1997. Proc Natl Acad Sci U S A 1998;95:5437-5441.

27. Boscoe FP, Schymura MJ. Solar ultraviolet-B exposure and cancer incidence and mortality in the United States, 1993-2002. BMC Cancer 2006;6:264.

28. Seidler A, Mohner M, Berger J, Mester B, Deeg E, Elsner G, Nieters A, Becker N. Solvent exposure and malignant lymphoma: a population-based case-control study in Germany. J Occup Med Toxicol 2007;2:2.

29. Rericha V, Kulich M, Rericha R, Shore DL, Sandler DP. Incidence of leukemia, lymphoma, and multiple myeloma in Czech uranium miners: a case-cohort study. Environ Health Perspect 2006;114:818-822.

30. Chiu BC, Dave BJ, Blair A, Gapstur SM, Zahm SH, Weisenburger DD. Agricultural pesticide use and risk of t(14;18)-defined subtypes of non-Hodgkin lymphoma. Blood 2006;108:1363-1369.

31. Linet MS, Yin SN, Travis LB, Li CY, Zhang ZN, Li DG, Rothman N, Li GL, Chow WH, Donaldson J, Dosemeci M, Wacholder S, Blot WJ, Hayes RB. Clinical features of hematopoietic malignancies and related disorders among benzene-exposed workers in China. Benzene Study Group. Environ Health Perspect 1996;104 Suppl 6:1353-1364.

32. Chiu BC, Dave BJ, Blair A, Gapstur SM, Chmiel JS, Fought AJ, Zahm SH, Weisenburger DD. Cigarette smoking, familial hematopoietic cancer, hair dye use, and risk of t(14;18)-defined subtypes of non-Hodgkin's lymphoma. Am J Epidemiol 2007;165:652-659.

33. Kasim K, Levallois P, Abdous B, Auger P, Johnson KC. Lifestyle factors and the risk of adult leukemia in Canada. Cancer Causes Control 2005;16:489-500.

34. Morton LM, Holford TR, Leaderer B, Boyle P, Zahm SH, Zhang Y, Flynn S, Tallini G, Zhang B, Owens PH, Zheng T. Cigarette smoking and risk of non-Hodgkin lymphoma subtypes among women. Br J Cancer 2003;89:2087-2092.

35. Pertovaara M, Pukkala E, Laippala P, Miettinen A, Pasternack A. A longitudinal cohort study of Finnish patients with primary Sjogren's syndrome: clinical, immunological, and epidemiological aspects. Ann Rheum Dis 2001;60:467-472.

36. Nalesnik MA, Jaffe R, Starzl TE, Demetris AJ, Porter K, Burnham JA, Makowka L, Ho M, Locker J. The pathology of posttransplant lymphoproliferative disorders occurring in the setting of cyclosporine A-prednisone immunosuppression. Am J Pathol 1988;133:173-192.

37. Askling J, Brandt L, Lapidus A, Karlen P, Bjorkholm M, Lofberg R, Ekbom A. Risk of haematopoietic cancer in patients with inflammatory bowel disease. Gut 2005;54:617-622.

38. Farrell RJ, Ang Y, Kileen P, O'Briain DS, Kelleher D, Keeling PW, Weir DG. Increased incidence of non-Hodgkin's lymphoma in inflammatory bowel disease patients on immunosuppressive therapy but overall risk is low. Gut 2000;47:514-519.

39. Baecklund E, Ekbom A, Sparen P, Feltelius N, Klareskog L. Disease activity and risk of lymphoma in patients with rheumatoid arthritis: nested case-control study Sunlight and non-Hodgkin's lymphoma: a population-based cohort study in Sweden. BMJ 1998;317:180-181.

40. Kelly C, Baird G, Foster H, Hosker H, Griffiths I. Prognostic significance of paraproteinaemia in rheumatoid arthritis. Ann Rheum Dis 1991;50:290-294.

41. Hawkins MM, Wilson LM, Stovall MA, Marsden HB, Potok MH, Kingston JE, Chessells JM. Epipodophyllotoxins, alkylating agents, and radiation and risk of secondary leukaemia after childhood cancer. BMJ 1992;304:951-958.

42. Kuper H, Adami HO, Trichopoulos D. Infections as a major preventable cause of human cancer. J Intern Med 2000;248:171-183.

43. Boccardo E, Villa LL. Viral origins of human cancer. Curr Med Chem 2007;14:2526-2539.

44. Mueller N. Overview: viral agents and cancer. Environ Health Perspect 1995;103 Suppl 8:259-261.

45. de SS, Bosch R, Schouten T, Verkuijlen S, Nieters A, Foretova L, Maynadie M, Cocco PL, Staines A, Becker N, Brennan P, Benavente Y, Boffetta P, Meijer CJ, Middeldorp JM. Epstein-Barr virus infection and risk of lymphoma: immunoblot analysis of antibody responses against EBV-related proteins in a large series of lymphoma subjects and matched controls. Int J Cancer 2007;121:1806-1812.

46. Ulcickas YM, Quesenberry CP, Jr., Guo D, Caldwell C, Wells K, Shan J, Sanders L, Skovron ML, Iloeje U, Manos MM. Incidence of non-Hodgkin's lymphoma among individuals with chronic hepatitis B virus infection. Hepatology 2007;46:107-112.

47. Engels EA, Chatterjee N, Cerhan JR, Davis S, Cozen W, Severson RK, Whitby D, Colt JS, Hartge P. Hepatitis C virus infection and non-Hodgkin lymphoma: results of the NCI-SEER multi-center case-control study. Int J Cancer 2004;111:76-80.

48. Nordstrom M, Hardell L, Lindstrom G, Wingfors H, Hardell K, Linde A. Concentrations of organochlorines related to titers to Epstein-Barr virus early antigen IgG as risk factors for hairy cell leukemia. Environ Health Perspect 2000;108:441-445.

49. Matsuoka M. Human T-cell leukemia virus type I (HTLV-I) infection and the onset of adult T-cell leukemia (ATL). Retrovirology 2005;2:27.

50. de SS, Goedert JJ, Marshall V, Bellas C, Benavente Y, Bosch R, Domingo A, Fernandez de SA, Servitje O, Whitby D. Risk of malignant lymphoma associated with human herpesvirus-8: a case-control study in Spain. Br J Cancer 2004;90:2145-2148.

51. Kuchinskaya E, Heyman M, Grander D, Linderholm M, Soderhall S, Zaritskey A, Nordgren A, Porwit-MacDonald A, Zueva E, Pawitan Y, Corcoran M, Nordenskjold M, Blennow E. Children and adults with acute lymphoblastic leukaemia have similar gene expression profiles. Eur J Haematol 2005;74:466-480.

52. Bassan R, Gatta G, Tondini C, Willemze R. Adult acute lymphoblastic leukaemia. Crit Rev Oncol Hematol 2004;50:223-261.

53. Pui CH, Relling MV, Downing JR. Acute lymphoblastic leukemia. N Engl J Med 2004;350:1535-1548.

54. Pui CH, Relling MV, Campana D, Evans WE. Childhood acute lymphoblastic leukemia. Rev Clin Exp Hematol 2002;6:161-180.

55. Castagnola C, Lunghi M, Caberlon S, Bonfichi M, Pascutto C, Lazzarino M. Long-term outcome of ph-negative acute lymphoblastic leukaemia in adults: a single centre experience. Acta Haematol 2005;113:234-240.

56. Faderl S, Thall PF, Kantarjian HM, Estrov Z. Time to platelet recovery predicts outcome of patients with de novo acute lymphoblastic leukaemia who have achieved a complete remission. Br J Haematol 2002;117:869-874.

57. De AG, Yuen C, Palla SL, Anderson PM, Zweidler-McKay PA. Absolute lymphocyte count is a novel prognostic indicator in ALL and AML: implications for risk stratification and future studies. Cancer 2008;112:407-415.

58. Maitra A, McKenna RW, Weinberg AG, Schneider NR, Kroft SH. Precursor B-cell lymphoblastic lymphoma. A study of nine cases lacking blood and bone marrow involvement and review of the literature. Am J Clin Pathol 2001;115:868-875.

59. Pui CH, Schrappe M, Ribeiro RC, Niemeyer CM. Childhood and adolescent lymphoid and myeloid leukemia. Hematology Am Soc Hematol Educ Program 2004;118-145.

60. Le QH, Thomas X, Ecochard R, Iwaz J, Lheritier V, Michallet M, Fiere D. Initial and late prognostic factors to predict survival in adult acute lymphoblastic leukaemia. Eur J Haematol 2006;77:471-479.

61. Baccarani M, Corbelli G, Amadori S, Drenthe-Schonk A, Willemze R, Meloni G, Cardozo PL, Haanen C, Mandelli F, Tura S. Adolescent and adult acute lymphoblastic leukemia: prognostic features and outcome of therapy. A study of 293 patients. Blood 1982;60:677-684.

62. Harris NL, Jaffe ES, Diebold J, Flandrin G, Muller-Hermelink HK, Vardiman J, Lister TA, Bloomfield CD. The World Health Organization classification of neoplasms of the hematopoietic and lymphoid tissues: report of the Clinical Advisory Committee meeting--Airlie House, Virginia, November, 1997. Hematol J 2000;1:53-66.

63. Wrzesien-Kus A, Robak T, Pluta A, Zwolinska M, Wawrzyniak E, Wierzbowska A, Skotnicki A, Jakubas B, Holowiecki J, Nowak K, Kuliczkowski K, Mazur G, Haus O, Dmoszynska A, mczyk-Cioch M, Jedrzejczak WW, Paluszewska M, Konopka L, Palynyczko G. Outcome of treatment in adults with Philadelphia chromosome-positive and/or BCR-ABL--positive acute lymphoblastic leukemia-retrospective analysis of Polish Adult Leukemia Group (PALG). Ann Hematol 2006;85:366-373.

64. Dombret H, Gabert J, Boiron JM, Rigal-Huguet F, Blaise D, Thomas X, Delannoy A, Buzyn A, Bilhou-Nabera C, Cayuela JM, Fenaux P, Bourhis JH, Fegueux N, Charrin C, Boucheix C, Lheritier V, Esperou H, MacIntyre E, Vernant JP, Fiere D. Outcome of treatment in adults with Philadelphia chromosome-positive acute lymphoblastic leukemia--results of the prospective multicenter LALA-94 trial. Blood 2002;100:2357-2366.

65. Moorman AV, Harrison CJ, Buck GA, Richards SM, Secker-Walker LM, Martineau M, Vance GH, Cherry AM, Higgins RR, Fielding AK, Foroni L, Paietta E, Tallman MS, Litzow MR, Wiernik PH, Rowe JM, Goldstone AH, Dewald GW. Karyotype is an independent prognostic factor in adult acute lymphoblastic leukemia (ALL): analysis of cytogenetic data from patients treated on the Medical Research Council (MRC) UKALLXII/Eastern Cooperative Oncology Group (ECOG) 2993 trial. Blood 2007;109:3189-3197.

66. Bodey GP, Rolston KV. Management of fever in neutropenic patients. J Infect Chemother 2001;7:1-9.

67. Rolston KV. Management of infections in the neutropenic patient. Annu Rev Med 2004;55:519-526.

68. Velasco E, Byington R, Martins CA, Schirmer M, Dias LM, Goncalves VM. Prospective evaluation of the epidemiology, microbiology, and outcome of bloodstream infections in hematologic patients in a single cancer center. Eur J Clin Microbiol Infect Dis 2003;22:137-143.

69. Velasco E, Byington R, Martins CA, Schirmer M, Dias LM, Goncalves VM. Comparative study of clinical characteristics of neutropenic and non-neutropenic adult cancer patients with bloodstream infections. Eur J Clin Microbiol Infect Dis 2006;25:1-7.

70. Wisplinghoff H, Cornely OA, Moser S, Bethe U, Stutzer H, Salzberger B, Fatkenheuer G, Seifert H. Outcomes of nosocomial bloodstream infections in adult neutropenic patients: a prospective cohort and matched case-control study. Infect Control Hosp Epidemiol 2003;24:905-911.

71. Raad I, Hanna H, Maki D. Intravascular catheter-related infections: advances in diagnosis, prevention, and management. Lancet Infect Dis 2007;7:645-657.

72. Donnelly JP. Infection in the neutropenic and haematopoietic stem cell transplant recipient. Curr Opin Infect Dis 2000;13:337-342.

73. Raad II, Hanna HA. Intravascular catheter-related infections: new horizons and recent advances. Arch Intern Med 2002;162:871-878.

74. Mermel LA. Prevention of intravascular catheter-related infections. Ann Intern Med 2000;132:391-402.

75. Seifert H, Cornely O, Seggewiss K, Decker M, Stefanik D, Wisplinghoff H, Fatkenheuer G. Bloodstream infection in neutropenic cancer patients related to short-term nontunnelled catheters determined by quantitative blood cultures, differential time to positivity, and molecular epidemiological typing with pulsed-field gel electrophoresis. J Clin Microbiol 2003;41:118-123.

76. Raad I, Hanna HA, Alakech B, Chatzinikolaou I, Johnson MM, Tarrand J. Differential time to positivity: a useful method for diagnosing catheter-related bloodstream infections. Ann Intern Med 2004;140:18-25.

77. Blot F, Nitenberg G, Chachaty E, Raynard B, Germann N, Antoun S, Laplanche A, Brun-Buisson C, Tancrede C. Diagnosis of catheter-related bacteraemia: a prospective comparison of the time to positivity of hub-blood versus peripheral-blood cultures. Lancet 1999;354:1071-1077.

78. Cohen J. The immunopathogenesis of sepsis. Nature 2002;420:885-891.

79. Schiel X, Hebart H, Kern WV, Kiehl MG, Solch JP, Wilhelm S, Ostermann H. Sepsis in neutropenia--guidelines of the Infectious Diseases Working Party (AGIHO) of the German Society of Hematology and Oncology (DGHO). Ann Hematol 2003;82 Suppl 2:S158-S166.

80. Martin GS, Mannino DM, Eaton S, Moss M. The epidemiology of sepsis in the United States from 1979 through 2000. N Engl J Med 2003;348:1546-1554.

81. von Lilienfeld-Toal M, Dietrich MP, Glasmacher A, Lehmann L, Breig P, Hahn C, Schmidt-Wolf IG, Marklein G, Schroeder S, Stuber F. Markers of bacteremia in febrile neutropenic patients with hematological malignancies: procalcitonin and IL-6 are more reliable than C-reactive protein. Eur J Clin Microbiol Infect Dis 2004;23:539-544.

82. Poyart C, Morand P, Buzyn A. [Etiology of bacterial infections in febrile neutropenic patients: the role of the laboratory in the diagnosis]. Presse Med 2004;33:460-466.

83. Maschmeyer G, Beinert T, Buchheidt D, Einsele H, Heussel CP, Kiehl M, Lorenz J. Diagnosis and antimicrobial therapy of pulmonary infiltrates in febrile neutropenic patients--guidelines of the Infectious Diseases Working Party (AGIHO) of the German Society of Hematology and Oncology (DGHO). Ann Hematol 2003;82 Suppl 2:S118-S126.

84. Maschmeyer G, Link H, Hiddemann W, Meyer P, Helmerking M, Eisenmann E, Schmitt J, Adam D. Pulmonary infiltrations in febrile patients with neutropenia. Risk factors and outcome under empirical antimicrobial therapy in a randomized multicenter study. Cancer 1994;73:2296-2304.

85. Maertens J, Verhaegen J, Demuynck H, Brock P, Verhoef G, Vandenberghe P, Van EJ, Verbist L, Boogaerts M. Autopsy-controlled prospective evaluation of serial screening for circulating galactomannan by a sandwich enzyme-linked immunosorbent assay for hematological patients at risk for invasive Aspergillosis. J Clin Microbiol 1999;37:3223-3228.

86. Picazo JJ. Management of the febrile neutropenic patient: a consensus conference. Clin Infect Dis 2004;39 Suppl 1:S1-S6.

87. O'Grady NP, Alexander M, Dellinger EP, Gerberding JL, Heard SO, Maki DG, Masur H, McCormick RD, Mermel LA, Pearson ML, Raad II, Randolph A, Weinstein RA. Guidelines for the prevention of intravascular catheter-related infections. Infect Control Hosp Epidemiol 2002;23:759-769.

88. Yoshida M, Ohno R. Antimicrobial prophylaxis in febrile neutropenia. Clin Infect Dis 2004;39 Suppl 1:S65-S67.

89. Garcia-Carbonero R, Mayordomo JI, Tornamira MV, Lopez-Brea M, Rueda A, Guillem V, Arcediano A, Yubero A, Ribera F, Gomez C, Tres A, Perez-Gracia JL, Lumbreras C, Hornedo J, Cortes-Funes H, Paz-Ares L. Granulocyte colony-stimulating factor in the treatment of high-risk febrile neutropenia: a multicenter randomized trial. J Natl Cancer Inst 2001;93:31-38.

90. Fluckiger U, Marchetti O, Bille J, Eggimann P, Zimmerli S, Imhof A, Garbino J, Ruef C, Pittet D, Tauber M, Glauser M, Calandra T. Treatment options of invasive fungal infections in adults. Swiss Med Wkly 2006;136:447-463.

91. Mermel LA, Farr BM, Sherertz RJ, Raad II, O'Grady N, Harris JS, Craven DE. Guidelines for the management of intravascular catheter-related infections. Clin Infect Dis 2001;32:1249-1272.

92. Kobayashi KS, Chamaillard M, Ogura Y, Henegariu O, Inohara N, Nunez G, Flavell RA. Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. Science 2005;307:731-734.

93. Inohara N, Nunez G. NODs: intracellular proteins involved in inflammation and apoptosis. Nat Rev Immunol 2003;3:371-382.

94. Takeda K, Akira S. Toll-like receptors in innate immunity. Int Immunol 2005;17:1-14.

95. Korzenik JR. Is Crohn's disease due to defective immunity? Gut 2007;56:2-5.

96. Lehrnbecher T, Bernig T, Hanisch M, Koehl U, Behl M, Reinhardt D, Creutzig U, Klingebiel T, Chanock SJ, Schwabe D. Common genetic variants in the interleukin-6 and chitotriosidase genes are associated with the risk for serious infection in children undergoing therapy for acute myeloid leukemia. Leukemia 2005;19:1745-1750.

97. van EM, Scheij SS, van Roomen CP, Speijer D, Boot RG, Aerts JM. TLR- and NOD2-dependent regulation of human phagocyte-specific chitotriosidase. FEBS Lett 2007;581:5389-5395.

98. Boot RG, Blommaart EF, Swart E, Ghauharali-van d, V, Bijl N, Moe C, Place A, Aerts JM. Identification of a novel acidic mammalian chitinase distinct from chitotriosidase. J Biol Chem 2001;276:6770-6778.

99. Boot RG, Renkema GH, Strijland A, van Zonneveld AJ, Aerts JM. Cloning of a cDNA encoding chitotriosidase, a human chitinase produced by macrophages. J Biol Chem 1995;270:26252-26256.

100. Hollak CE, van WS, van Oers MH, Aerts JM. Marked elevation of plasma chitotriosidase activity. A novel hallmark of Gaucher disease. J Clin Invest 1994;93:1288-1292.

101. Fusetti F, von MH, Houston D, Rozeboom HJ, Dijkstra BW, Boot RG, Aerts JM, van Aalten DM. Structure of human chitotriosidase. Implications for specific inhibitor design and function of mammalian chitinase-like lectins. J Biol Chem 2002;277:25537-25544.

102. Malaguarnera L, Simpore J, Prodi DA, Angius A, Sassu A, Persico I, Barone R, Musumeci S. A 24-bp duplication in exon 10 of human chitotriosidase gene from the sub-Saharan to the Mediterranean area: role of parasitic diseases and environmental conditions. Genes Immun 2003;4:570-574.

103. Boot RG, Bussink AP, Verhoek M, de Boer PA, Moorman AF, Aerts JM. Marked differences in tissue-specific expression of chitinases in mouse and man. J Histochem Cytochem 2005;53:1283-1292.

104. Malaguarnera L, Di RM, Zambito AM, dell'Ombra N, Nicoletti F, Malaguarnera M. Chitotriosidase gene expression in Kupffer cells from patients with non-alcoholic fatty liver disease. Gut 2006;55:1313-1320.

105. Di RM, dell'Ombra N, Zambito AM, Malaguarnera M, Nicoletti F, Malaguarnera L. Chitotriosidase and inflammatory mediator levels in Alzheimer's disease and cerebrovascular dementia. Eur J Neurosci 2006;23:2648-2656.

106. Boot RG, Renkema GH, Verhoek M, Strijland A, Bliek J, de Meulemeester TM, Mannens MM, Aerts JM. The human chitotriosidase gene. Nature of inherited enzyme deficiency. J Biol Chem 1998;273:25680-25685.

107. Rodrigues MR, Sa Miranda MC, Amaral O. Allelic frequency determination of the 24-bp chitotriosidase duplication in the Portuguese population by real-time PCR. Blood Cells Mol Dis 2004;33:362-364.

108. Lee P, Waalen J, Crain K, Smargon A, Beutler E. Human chitotriosidase polymorphisms G354R and A442V associated with reduced enzyme activity. Blood Cells Mol Dis 2007;39:353-360.

109. Malaguarnera L, Musumeci M, Di RM, Scuto A, Musumeci S. Interferon-gamma, tumor necrosis factor-alpha, and lipopolysaccharide promote chitotriosidase gene expression in human macrophages. J Clin Lab Anal 2005;19:128-132.

110. Lauw FN, te Velde AA, Dekkers PE, Speelman P, Aerts JM, Hack CE, van Deventer SJ, van der PT. Activation of mononuclear cells by interleukin-12: an in vivo study in chimpanzees. J Clin Immunol 1999;19:231-238.

111. Romani L, Puccetti P, Bistoni F. Interleukin-12 in infectious diseases. Clin Microbiol Rev 1997;10:611-636.

112. Barone R, Simpore J, Malaguarnera L, Pignatelli S, Musumeci S. Plasma chitotriosidase activity in acute Plasmodium falciparum malaria. Clin Chim Acta 2003;331:79-85.

113. Malaguarnera L, Imbesi RM, Pignatelli S, Simpore J, Malaguarnera M, Musumeci S. Increased levels of interleukin-12 in Plasmodium falciparum malaria: correlation with the severity of disease. Parasite Immunol 2002;24:387-389.

114. Malaguarnera L, Musumeci M, Licata F, Di RM, Messina A, Musumeci S. Prolactin induces chitotriosidase gene expression in human monocyte-derived macrophages. Immunol Lett 2004;94:57-63.

115. Choi EH, Zimmerman PA, Foster CB, Zhu S, Kumaraswami V, Nutman TB, Chanock SJ. Genetic polymorphisms in molecules of innate immunity and susceptibility to infection with Wuchereria bancrofti in South India. Genes Immun 2001;2:248-253.

116. Guggenbuhl P, Grosbois B, Chales G. Gaucher disease. Joint Bone Spine 2008;75:116-124.

117. Hollak CE, Maas M, Aerts JM. Clinically relevant therapeutic endpoints in type I Gaucher disease. J Inherit Metab Dis 2001;24 Suppl 2:97-105.

118. Wajner A, Michelin K, Burin MG, Pires RF, Pereira ML, Giugliani R, Coelho JC. Comparison between the biochemical properties of plasma chitotriosidase from normal individuals and from patients with Gaucher disease, GM1-gangliosidosis, Krabbe disease and heterozygotes for Gaucher disease. Clin Biochem 2007;40:365-369.

119. Bierbaum S, Nickel R, Koch A, Lau S, Deichmann KA, Wahn U, Superti-Furga A, Heinzmann A. Polymorphisms and haplotypes of acid mammalian chitinase are associated with bronchial asthma. Am J Respir Crit Care Med 2005;172:1505-1509.

120. Bierbaum S, Superti-Furga A, Heinzmann A. Genetic polymorphisms of chitotriosidase in Caucasian children with bronchial asthma. Int J Immunogenet 2006;33:201-204.

121. Brunner J, Scholl-Burgi S, Prelog M, Zimmerhackl LB. Chitotriosidase as a marker of disease activity in sarcoidosis. Rheumatol Int 2007;27:1185-1186.

122. Barone R, Di GF, Romeo MA, Schiliro G, Pavone L. Plasma chitotriosidase activity in patients with beta-thalassemia. Blood Cells Mol Dis 1999;25:1-8.

123. Karadag B, Kucur M, Isman FK, Hacibekiroglu M, Vural VA. Serum chitotriosidase activity in patients with coronary artery disease. Circ J 2008;72:71-75.

124. Artieda M, Cenarro A, Ganan A, Jerico I, Gonzalvo C, Casado JM, Vitoria I, Puzo J, Pocovi M, Civeira F. Serum chitotriosidase activity is increased in subjects with atherosclerosis disease. Arterioscler Thromb Vasc Biol 2003;23:1645-1652.

125. Sotgiu S, Barone R, Zanda B, Arru G, Fois ML, Arru A, Rosati G, Marchetti B, Musumeci S. Chitotriosidase in patients with acute ischemic stroke. Eur Neurol 2005;54:149-153.

126. Sotgiu S, Barone R, Arru G, Fois ML, Pugliatti M, Sanna A, Rosati G, Musumeci S. Intrathecal chitotriosidase and the outcome of multiple sclerosis. Mult Scler 2006;12:551-557.

127. Vedder AC, Cox-Brinkman J, Hollak CE, Linthorst GE, Groener JE, Helmond MT, Scheij S, Aerts JM. Plasma chitotriosidase in male Fabry patients: a marker for monitoring lipid-laden macrophages and their correction by enzyme replacement therapy. Mol Genet Metab 2006;89:239-244.

128. Masoud M, Rudensky B, Elstein D, Zimran A. Chitotriosidase deficiency in survivors of Candida sepsis. Blood Cells Mol Dis 2002;29:116-118.

129. Hugot JP, Laurent-Puig P, Gower-Rousseau C, Olson JM, Lee JC, Beaugerie L, Naom I, Dupas JL, Van GA, Orholm M, Bonaiti-Pellie C, Weissenbach J, Mathew CG, Lennard-Jones JE, Cortot A, Colombel JF, Thomas G. Mapping of a susceptibility locus for Crohn's disease on chromosome 16. Nature 1996;379:821-823.

130. Hugot JP, Chamaillard M, Zouali H, Lesage S, Cezard JP, Belaiche J, Almer S, Tysk C, O'Morain CA, Gassull M, Binder V, Finkel Y, Cortot A, Modigliani R, Laurent-Puig P, Gower-Rousseau C, Macry J, Colombel JF, Sahbatou M, Thomas G. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. Nature 2001;411:599-603.

131. Ogura Y, Inohara N, Benito A, Chen FF, Yamaoka S, Nunez G. Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-kappaB. J Biol Chem 2001;276:4812-4818.

132. Inohara, Chamaillard, McDonald C, Nunez G. NOD-LRR proteins: role in host-microbial interactions and inflammatory disease. Annu Rev Biochem 2005;74:355-383.

133. Martin SJ. Dealing the CARDs between life and death. Trends Cell Biol 2001;11:188-189.

134. Abraham C, Cho JH. Functional consequences of NOD2 (CARD15) mutations. Inflamm Bowel Dis 2006;12:641-650.

135. Inohara N, Koseki T, Lin J, del PL, Lucas PC, Chen FF, Ogura Y, Nunez G. An induced proximity model for NF-kappa B activation in the Nod1/RICK and RIP signaling pathways. J Biol Chem 2000;275:27823-27831.

136. Inohara N, Koseki T, del PL, Hu Y, Yee C, Chen S, Carrio R, Merino J, Liu D, Ni J, Nunez G. Nod1, an Apaf-1-like activator of caspase-9 and nuclear factor-kappaB. J Biol Chem 1999;274:14560-14567.

137. Inohara N, Ogura Y, Fontalba A, Gutierrez O, Pons F, Crespo J, Fukase K, Inamura S, Kusumoto S, Hashimoto M, Foster SJ, Moran AP, Fernandez-Luna JL, Nunez G. Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. J Biol Chem 2003;278:5509-5512.

138. Inohara N, Ogura Y, Chen FF, Muto A, Nunez G. Human Nod1 confers responsiveness to bacterial lipopolysaccharides. J Biol Chem 2001;276:2551-2554.

139. Girardin SE, Boneca IG, Viala J, Chamaillard M, Labigne A, Thomas G, Philpott DJ, Sansonetti PJ. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. J Biol Chem 2003;278:8869-8872.

140. Rosenstiel P, Fantini M, Brautigam K, Kuhbacher T, Waetzig GH, Seegert D, Schreiber S. TNF-alpha and IFN-gamma regulate the expression of the NOD2 (CARD15) gene in human intestinal epithelial cells. Gastroenterology 2003;124:1001-1009.

141. Kobayashi K, Inohara N, Hernandez LD, Galan JE, Nunez G, Janeway CA, Medzhitov R, Flavell RA. RICK/Rip2/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems. Nature 2002;416:194-199.

142. Watanabe T, Kitani A, Murray PJ, Strober W. NOD2 is a negative regulator of Toll-like receptor 2-mediated T helper type 1 responses. Nat Immunol 2004;5:800-808.

143. Inohara N, Nunez G. The NOD: a signaling module that regulates apoptosis and host defense against pathogens. Oncogene 2001;20:6473-6481.

144. Gutierrez O, Pipaon C, Inohara N, Fontalba A, Ogura Y, Prosper F, Nunez G, Fernandez-Luna JL. Induction of Nod2 in myelomonocytic and intestinal epithelial cells via nuclear factor-kappa B activation. J Biol Chem 2002;277:41701-41705.

145. Lala S, Ogura Y, Osborne C, Hor SY, Bromfield A, Davies S, Ogunbiyi O, Nunez G, Keshav S. Crohn's disease and the NOD2 gene: a role for paneth cells. Gastroenterology 2003;125:47-57.

146. Hugot JP, Zaccaria I, Cavanaugh J, Yang H, Vermeire S, Lappalainen M, Schreiber S, Annese V, Jewell DP, Fowler EV, Brant SR, Silverberg MS, Cho J, Rioux JD, Satsangi J, Parkes M. Prevalence of CARD15/NOD2 mutations in Caucasian healthy people. Am J Gastroenterol 2007;102:1259-1267.

147. Cavanaugh J. NOD2: ethnic and geographic differences. World J Gastroenterol 2006;12:3673-3677.

148. Economou M, Trikalinos TA, Loizou KT, Tsianos EV, Ioannidis JP. Differential effects of NOD2 variants on Crohn's disease risk and phenotype in diverse populations: a metaanalysis. Am J Gastroenterol 2004;99:2393-2404.

149. Cuthbert AP, Fisher SA, Mirza MM, King K, Hampe J, Croucher PJ, Mascheretti S, Sanderson J, Forbes A, Mansfield J, Schreiber S, Lewis CM, Mathew CG. The contribution of NOD2 gene mutations to the risk and site of disease in inflammatory bowel disease. Gastroenterology 2002;122:867-874.

150. Gasche C, Nemeth M, Grundtner P, Willheim-Polli C, Ferenci P, Schwarzenbacher R. Evolution of Crohn's disease-associated Nod2 mutations. Immunogenetics 2008;60:115-120.

151. Hampe J, Frenzel H, Mirza MM, Croucher PJ, Cuthbert A, Mascheretti S, Huse K, Platzer M, Bridger S, Meyer B, Nurnberg P, Stokkers P, Krawczak M, Mathew CG, Curran M, Schreiber S. Evidence for a NOD2-independent susceptibility locus for inflammatory bowel disease on chromosome 16p. Proc Natl Acad Sci U S A 2002;99:321-326.

152. Lesage S, Zouali H, Cezard JP, Colombel JF, Belaiche J, Almer S, Tysk C, O'Morain C, Gassull M, Binder V, Finkel Y, Modigliani R, Gower-Rousseau C, Macry J, Merlin F, Chamaillard M, Jannot AS, Thomas G, Hugot JP. CARD15/NOD2 mutational analysis and genotype-phenotype correlation in 612 patients with inflammatory bowel disease. Am J Hum Genet 2002;70:845-857.

153. Ahmad T, Armuzzi A, Bunce M, Mulcahy-Hawes K, Marshall SE, Orchard TR, Crawshaw J, Large O, de SA, Cook JT, Barnardo M, Cullen S, Welsh KI, Jewell DP. The molecular classification of the clinical manifestations of Crohn's disease. Gastroenterology 2002;122:854-866.

154. Kanazawa N, Okafuji I, Kambe N, Nishikomori R, Nakata-Hizume M, Nagai S, Fuji A, Yuasa T, Manki A, Sakurai Y, Nakajima M, Kobayashi H, Fujiwara I, Tsutsumi H, Utani A, Nishigori C, Heike T, Nakahata T, Miyachi Y. Early-onset sarcoidosis and CARD15 mutations with constitutive nuclear factor-kappaB activation: common genetic etiology with Blau syndrome. Blood 2005;105:1195-1197.

155. Miceli-Richard C, Lesage S, Rybojad M, Prieur AM, Manouvrier-Hanu S, Hafner R, Chamaillard M, Zouali H, Thomas G, Hugot JP. CARD15 mutations in Blau syndrome. Nat Genet 2001;29:19-20.

156. Kurzawski G, Suchy J, Kladny J, Grabowska E, Mierzejewski M, Jakubowska A, Debniak T, Cybulski C, Kowalska E, Szych Z, Domagala W, Scott RJ, Lubinski J. The NOD2 3020insC mutation and the risk of colorectal cancer. Cancer Res 2004;64:1604-1606.

157. Suchy J, Klujszo-Grabowska E, Kladny J, Cybulski C, Wokolorczyk D, Szymanska-Pasternak J, Kurzawski G, Scott RJ, Lubinski J. Inflammatory response gene polymorphisms and their relationship with colorectal cancer risk. BMC Cancer 2008;8:112.

158. Tuupanen S, Alhopuro P, Mecklin JP, Jarvinen H, Aaltonen LA. No evidence for association of NOD2 R702W and G908R with colorectal cancer. Int J Cancer 2007;121:76-79.

159. Rosenstiel P, Hellmig S, Hampe J, Ott S, Till A, Fischbach W, Sahly H, Lucius R, Folsch UR, Philpott D, Schreiber S. Influence of polymorphisms in the NOD1/CARD4 and NOD2/CARD15 genes on the clinical outcome of Helicobacter pylori infection. Cell Microbiol 2006;8:1188-1198.

160. Lener MR, Oszutowska D, Castaneda J, Kurzawski G, Suchy J, Nej-Wolosiak K, Byrski T, Huzarski T, Gronwald J, Szymanska A, Szymanska-Pasternak J, Grodzki T, Serwatowski P, Bre BG, Scott RJ, Lubinski J. Prevalence of the NOD2 3020insC mutation in aggregations of breast and lung cancer. Breast Cancer Res Treat 2006;95:141-145.

161. Huzarski T, Lener M, Domagala W, Gronwald J, Byrski T, Kurzawski G, Suchy J, Chosia M, Woyton J, Ucinski M, Narod SA, Lubinski J. The 3020insC allele of NOD2 predisposes to early-onset breast cancer. Breast Cancer Res Treat 2005;89:91-93.

162. Forrest MS, Skibola CF, Lightfoot TJ, Bracci PM, Willett EV, Smith MT, Holly EA, Roman E. Polymorphisms in innate immunity genes and risk of non-Hodgkin lymphoma. Br J Haematol 2006;134:180-183.

163. Ahrens P, Kattner E, Kohler B, Hartel C, Seidenberg J, Segerer H, Moller J, Gopel W. Mutations of genes involved in the innate immune system as predictors of sepsis in very low birth weight infants. Pediatr Res 2004;55:652-656.

164. Brenmoehl J, Herfarth H, Gluck T, Audebert F, Barlage S, Schmitz G, Froehlich D, Schreiber S, Hampe J, Scholmerich J, Holler E, Rogler G. Genetic variants in the NOD2/CARD15 gene are associated with early mortality in sepsis patients. Intensive Care Med 2007;33:1541-1548.

165. Rahman P, Bartlett S, Siannis F, Pellett FJ, Farewell VT, Peddle L, Schentag CT, Alderdice CA, Hamilton S, Khraishi M, Tobin Y, Hefferton D, Gladman DD. CARD15: a pleiotropic autoimmune gene that confers susceptibility to psoriatic arthritis. Am J Hum Genet 2003;73:677-681.

166. Kabesch M, Peters W, Carr D, Leupold W, Weiland SK, von ME. Association between polymorphisms in caspase recruitment domain containing protein 15 and allergy in two German populations. J Allergy Clin Immunol 2003;111:813-817.

167. El Mokhtari NE, Ott SJ, Nebel A, Schafer A, Rosenstiel P, Forster M, Nothnagel M, Simon R, Schreiber S. Role of NOD2/CARD15 in coronary heart disease. BMC Genet 2007;8:76.

168. Ferreiros-Vidal I, Garcia-Meijide J, Carreira P, Barros F, Carracedo A, Gomez-Reino JJ, Gonzalez A. The three most common CARD15 mutations associated with Crohn's disease and the chromosome 16 susceptibility locus for systemic lupus erythematosus. Rheumatology (Oxford) 2003;42:570-574.

169. Ahmad T, Zhang L, Gogus F, Verity D, Wallace G, Madanat W, Fayyad F, James T, Neville M, Kanawati C, Fortune F, Celik A, Stanford M, Jewell DP, Marshall SE. CARD15 polymorphisms in Behcet's disease. Scand J Rheumatol 2005;34:233-237.

170. Sawcer S, Maranian M, Hensiek A, Roxburgh R, Gray J, Compston A. Crohn's associated NOD2 gene variants are not involved in determining susceptibility to multiple sclerosis. J Neurol Neurosurg Psychiatry 2003;74:1157.

171. van der PM, Crusius JB, de Koning MH, Murillo LS, van de Stadt RJ, Dijkmans BA, Pena AS, van der Horst-Bruinsma IE. CARD15 gene mutations are not associated with ankylosing spondylitis. Genes Immun 2003;4:77-78.

172. Ferreiros-Vidal I, Barros F, Pablos JL, Carracedo A, Gomez-Reino JJ, Gonzalez A. CARD15/NOD2 analysis in rheumatoid arthritis susceptibility. Rheumatology (Oxford) 2003;42:1380-1382.

173. Stockton JC, Howson JM, Awomoyi AA, McAdam KP, Blackwell JM, Newport MJ. Polymorphism in NOD2, Crohn's disease, and susceptibility to pulmonary tuberculosis. FEMS Immunol Med Microbiol 2004;41:157-160.

174. Laine ML, Murillo LS, Morre SA, Winkel EG, Pena AS, van Winkelhoff AJ. CARD15 gene mutations in periodontitis. J Clin Periodontol 2004;31:890-893.

175. Henckaerts L, Nielsen KR, Steffensen R, Van SK, Mathieu C, Giulietti A, Wouters PJ, Milants I, Vanhorebeek I, Langouche L, Vermeire S, Rutgeerts P, Thiel S, Wilmer A, Hansen TK, Van den BG. Polymorphisms in innate immunity genes predispose to bacteremia and death in the medical intensive care unit. Crit Care Med 2009;37:192-193.

176. Henckaerts L, Vermeire S. NOD2/CARD15 disease associations other than Crohn's disease. Inflamm Bowel Dis 2007;13:235-241.

177. Weidinger S, Klopp N, Rummler L, Wagenpfeil S, Baurecht HJ, Gauger A, Darsow U, Jakob T, Novak N, Schafer T, Heinrich J, Behrendt H, Wichmann HE, Ring J, Illig T. Association of CARD15 polymorphisms with atopy-related traits in a population-based cohort of Caucasian adults. Clin Exp Allergy 2005;35:866-872.

178. Cho JH, Fraser IP, Fukase K, Kusumoto S, Fujimoto Y, Stahl GL, Ezekowitz RA. Human peptidoglycan recognition protein S is an effector of neutrophil-mediated innate immunity. Blood 2005;106:2551-2558.

179. Borregaard N, Sorensen OE, Theilgaard-Monch K. Neutrophil granules: a library of innate immunity proteins. Trends Immunol 2007;28:340-345.

180. Coyle LA, Papaioannou M, Yaxley JC, Chim JS, Attard M, Hoffbrand AV, Foroni L. Molecular analysis of the leukaemic B cell in adult and childhood acute lymphoblastic leukaemia. Br J Haematol 1996;94:685-693.

181. Escott GM, Adams DJ. Chitinase activity in human serum and leukocytes. Infect Immun 1995;63:4770-4773.

182. Aguilera B, Ghauharali-van d, V, Helmond MT, Out JM, Donker-Koopman WE, Groener JE, Boot RG, Renkema GH, van der Marel GA, van Boom JH, Overkleeft HS, Aerts JM. Transglycosidase activity of chitotriosidase: improved enzymatic assay for the human macrophage chitinase. J Biol Chem 2003;278:40911-40916.

183. De PB, Walsh TJ, Donnelly JP, Stevens DA, Edwards JE, Calandra T, Pappas PG, Maertens J, Lortholary O, Kauffman CA, Denning DW, Patterson TF, Maschmeyer G, Bille J, Dismukes WE, Herbrecht R, Hope WW, Kibbler CC, Kullberg BJ, Marr KA, Munoz P, Odds FC, Perfect JR, Restrepo A, Ruhnke M, Segal BH, Sobel JD, Sorrell TC, Viscoli C, Wingard JR, Zaoutis T, Bennett JE. Revised definitions of invasive fungal disease from the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group. Clin Infect Dis 2008;46:1813-1821.

184. Kyle RA, Rajkumar SV. Multiple myeloma. Blood 2008;111:2962-2972.

185. Morton LM, Wang SS, Devesa SS, Hartge P, Weisenburger DD, Linet MS. Lymphoma incidence patterns by WHO subtype in the United States, 1992-2001. Blood 2006;107:265-276.

186. Caggiano V, Weiss RV, Rickert TS, Linde-Zwirble WT. Incidence, cost, and mortality of neutropenia hospitalization associated with chemotherapy. Cancer 2005;103:1916-1924.

187. Link H, Maschmeyer G, Meyer P, Hiddemann W, Stille W, Helmerking M, Adam D. Interventional antimicrobial therapy in febrile neutropenic patients. Study Group of the Paul Ehrlich Society for Chemotherapy. Ann Hematol 1994;69:231-243.

188. Rolston KV. The spectrum of pulmonary infections in cancer patients. Curr Opin Oncol 2001;13:218-223.

189. Rolston KV, Bodey GP, Safdar A. Polymicrobial infection in patients with cancer: an underappreciated and underreported entity. Clin Infect Dis 2007;45:228-233.

190. Brown LM, Gridley G, Check D, Landgren O. Risk of multiple myeloma and monoclonal gammopathy of undetermined significance among white and black male United States veterans with prior autoimmune, infectious, inflammatory, and allergic disorders. Blood 2008;111:3388-3394.

191. Craig M, Cumpston AD, Hobbs GR, Devetten MP, Sarwari AR, Ericson SG. The clinical impact of antibacterial prophylaxis and cycling antibiotics for febrile neutropenia in a hematological malignancy and transplantation unit. Bone Marrow Transplant 2007;39:477-482.

192. Lever A, Mackenzie I. Sepsis: definition, epidemiology, and diagnosis. BMJ 2007;335:879-883.

193. Holmes CL, Russell JA, Walley KR. Genetic polymorphisms in sepsis and septic shock: role in prognosis and potential for therapy. Chest 2003;124:1103-1115.

194. Inohara N, Ogura Y, Nunez G. Nods: a family of cytosolic proteins that regulate the host response to pathogens. Curr Opin Microbiol 2002;5:76-80.

195. Hisamatsu T, Suzuki M, Reinecker HC, Nadeau WJ, McCormick BA, Podolsky DK. CARD15/NOD2 functions as an antibacterial factor in human intestinal epithelial cells. Gastroenterology 2003;124:993-1000.

196. Girardin SE, Hugot JP, Sansonetti PJ. Lessons from Nod2 studies: towards a link between Crohn's disease and bacterial sensing. Trends Immunol 2003;24:652-658.

197. Podolsky DK. Inflammatory bowel disease. N Engl J Med 2002;347:417-429.

198. Yamamoto-Furusho JK, Podolsky DK. Innate immunity in inflammatory bowel disease. World J Gastroenterol 2007;13:5577-5580.

199. Segal AW, Loewi G. Neutrophil dysfunction in Crohn's disease. Lancet 1976;2:219-221.

200. Curran FT, Allan RN, Keighley MR. Superoxide production by Crohn's disease neutrophils. Gut 1991;32:399-402.

201. Caradonna L, Amati L, Lella P, Jirillo E, Caccavo D. Phagocytosis, killing, lymphocyte-mediated antibacterial activity, serum autoantibodies, and plasma endotoxins in inflammatory bowel disease. Am J Gastroenterol 2000;95:1495-1502.

202. Marks DJ, Harbord MW, MacAllister R, Rahman FZ, Young J, Al-Lazikani B, Lees W, Novelli M, Bloom S, Segal AW. Defective acute inflammation in Crohn's disease: a clinical investigation. Lancet 2006;367:668-678.

203. Zhou Z, Lin XY, Akolkar PN, Gulwani-Akolkar B, Levine J, Katz S, Silver J. Variation at NOD2/CARD15 in familial and sporadic cases of Crohn's disease in the Ashkenazi Jewish population. Am J Gastroenterol 2002;97:3095-3101.

204. Suchy J, Klujszo-Grabowska E, Kladny J, Cybulski C, Wokolorczyk D, Szymanska-Pasternak J, Kurzawski G, Scott RJ, Lubinski J. Inflammatory response gene polymorphisms and their relationship with colorectal cancer risk. BMC Cancer 2008;8:112.

205. Kurzawski G, Suchy J, Kladny J, Grabowska E, Mierzejewski M, Jakubowska A, Debniak T, Cybulski C, Kowalska E, Szych Z, Domagala W, Scott RJ, Lubinski J. The NOD2 3020insC mutation and the risk of colorectal cancer. Cancer Res 2004;64:1604-1606.

206. Huzarski T, Lener M, Domagala W, Gronwald J, Byrski T, Kurzawski G, Suchy J, Chosia M, Woyton J, Ucinski M, Narod SA, Lubinski J. The 3020insC allele of NOD2 predisposes to early-onset breast cancer. Breast Cancer Res Treat 2005;89:91-93.

207. Lener MR, Oszutowska D, Castaneda J, Kurzawski G, Suchy J, Nej-Wolosiak K, Byrski T, Huzarski T, Gronwald J, Szymanska A, Szymanska-Pasternak J, Grodzki T, Serwatowski P, Bre BG, Scott RJ, Lubinski J. Prevalence of the NOD2 3020insC mutation in aggregations of breast and lung cancer. Breast Cancer Res Treat 2006;95:141-145.

208. Forrest MS, Skibola CF, Lightfoot TJ, Bracci PM, Willett EV, Smith MT, Holly EA, Roman E. Polymorphisms in innate immunity genes and risk of non-Hodgkin lymphoma. Br J Haematol 2006;134:180-183.

209. Rosenstiel P, Hellmig S, Hampe J, Ott S, Till A, Fischbach W, Sahly H, Lucius R, Folsch UR, Philpott D, Schreiber S. Influence of polymorphisms in the NOD1/CARD4 and NOD2/CARD15 genes on the clinical outcome of Helicobacter pylori infection. Cell Microbiol 2006;8:1188-1198.

210. Rolston KV. Management of infections in the neutropenic patient. Annu Rev Med 2004;55:519-526.

211. Caggiano V, Weiss RV, Rickert TS, Linde-Zwirble WT. Incidence, cost, and mortality of neutropenia hospitalization associated with chemotherapy. Cancer 2005;103:1916-1924.

212. Abraham C, Cho JH. Functional consequences of NOD2 (CARD15) mutations. Inflamm Bowel Dis 2006;12:641-650.

213. Schiel X, Hebart H, Kern WV, Kiehl MG, Solch JP, Wilhelm S, Ostermann H. Sepsis in neutropenia--guidelines of the Infectious Diseases Working Party (AGIHO) of the German Society of Hematology and Oncology (DGHO). Ann Hematol 2003;82 Suppl 2:S158-S166.

214. Link H, Maschmeyer G, Meyer P, Hiddemann W, Stille W, Helmerking M, Adam D. Interventional antimicrobial therapy in febrile neutropenic patients. Study Group of the Paul Ehrlich Society for Chemotherapy. Ann Hematol 1994;69:231-243.

215. Rolston KV. The spectrum of pulmonary infections in cancer patients. Curr Opin Oncol 2001;13:218-223.

216. Rolston KV, Bodey GP, Safdar A. Polymicrobial infection in patients with cancer: an underappreciated and underreported entity. Clin Infect Dis 2007;45:228-233.

217. Bonen DK, Ogura Y, Nicolae DL, Inohara N, Saab L, Tanabe T, Chen FF, Foster SJ, Duerr RH, Brant SR, Cho JH, Nunez G. Crohn's disease-associated NOD2 variants share a signaling defect in response to lipopolysaccharide and peptidoglycan. Gastroenterology 2003;124:140-146.

218. Abraham C, Cho JH. Functional consequences of NOD2 (CARD15) mutations. Inflamm Bowel Dis 2006;12:641-650.

219. Bonen DK, Ogura Y, Nicolae DL, Inohara N, Saab L, Tanabe T, Chen FF, Foster SJ, Duerr RH, Brant SR, Cho JH, Nunez G. Crohn's disease-associated NOD2 variants

share a signaling defect in response to lipopolysaccharide and peptidoglycan. Gastroenterology 2003;124:140-146.

220. Granell M, Urbano-Ispizua A, Arostegui JI, Fernandez-Aviles F, Martinez C, Rovira M, Rius J, Plaza S, Gaya A, Navarro A, Talarn C, Carreras E, Monzo M, Montserrat E, Yague J. Effect of NOD2/CARD15 variants in T-cell depleted allogeneic stem cell transplantation. Haematologica 2006;91:1372-1376.

221. Holler E, Rogler G, Brenmoehl J, Hahn J, Herfarth H, Greinix H, Dickinson AM, Socie G, Wolff D, Fischer G, Jackson G, Rocha V, Steiner B, Eissner G, Marienhagen J, Schoelmerich J, Andreesen R. Prognostic significance of NOD2/CARD15 variants in HLA-identical sibling hematopoietic stem cell transplantation: effect on long-term outcome is confirmed in 2 independent cohorts and may be modulated by the type of gastrointestinal decontamination. Blood 2006;107:4189-4193.

222. Kurzawski G, Suchy J, Kladny J, Grabowska E, Mierzejewski M, Jakubowska A, Debniak T, Cybulski C, Kowalska E, Szych Z, Domagala W, Scott RJ, Lubinski J. The NOD2 3020insC mutation and the risk of colorectal cancer. Cancer Res 2004;64:1604-1606.

223. Girardin SE, Travassos LH, Herve M, Blanot D, Boneca IG, Philpott DJ, Sansonetti PJ, Mengin-Lecreulx D. Peptidoglycan molecular requirements allowing detection by Nod1 and Nod2. J Biol Chem 2003;278:41702-41708.

224. van EM, van Roomen CP, Renkema GH, Bussink AP, Andrews L, Blommaart EF, Sugar A, Verhoeven AJ, Boot RG, Aerts JM. Characterization of human phagocyte-derived chitotriosidase, a component of innate immunity. Int Immunol 2005;17:1505-1512.

225. Bargagli E, Maggiorelli C, Rottoli P. Human Chitotriosidase: A Potential New Marker of Sarcoidosis Severity. Respiration 2008.

226. Iyer A, van EM, Silva E, Hatta M, Faber W, Aerts JM, Das PK. Increased chitotriosidase activity in serum of leprosy patients: association with bacillary leprosy. Clin Immunol 2009;131:501-509.

227. Bouzas L, San JE, Tutor JC. Chitotriosidase activity in pleural effusions. Clin Lab 2007;53:449-452.

228. Fishbein T, Novitskiy G, Mishra L, Matsumoto C, Kaufman S, Goyal S, Shetty K, Johnson L, Lu A, Wang A, Hu F, Kallakury B, Lough D, Zasloff M. NOD2-expressing bone marrow-derived cells appear to regulate epithelial innate immunity of the transplanted human small intestine. Gut 2008;57:323-330.

229. Appenrodt B, Grunhage F, Gentemann MG, Thyssen L, Sauerbruch T, Lammert F. Nucleotide-binding oligomerization domain containing 2 (NOD2) variants are genetic risk factors for death and spontaneous bacterial peritonitis in liver cirrhosis. Hepatology 2010;51:1327-1333.

230. Bonen DK, Ogura Y, Nicolae DL, Inohara N, Saab L, Tanabe T, Chen FF, Foster SJ, Duerr RH, Brant SR, Cho JH, Nunez G. Crohn's disease-associated NOD2 variants share a signaling defect in response to lipopolysaccharide and peptidoglycan. Gastroenterology 2003;124:140-146.

231. Mayor NP, Shaw BE, Keshav S, Madrigal JA, Marsh SG. A novel technique for NOD2/CARD15 genotyping using PCR-SSP. J Immunol Methods 2007;327:82-87.