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# **AETIOLOGY AND INFECTION SUSCEPTIBILITY IN NEUTROPAENIC PATIENTS**

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## ABSTRACT

Increasingly intensive antineoplastic regimes have been effective in the treatment for haematological malignancies, but as effective as it is in limiting neoplasm of the malignant cells, bystander damage occurs equally to cells of the immune system and epithelial cells of the mucosa. Infectious complications following chemotherapy reflect this situation.

The relationship between leukocytopaenia and infection risk, in the form of bacteraemia and fungaemia was first suggested by Bodey and colleagues and since then, many studies have followed establishing the breadth of pathogens in relation to neutropaenia. However, lymphocytes are also concurrently, markedly reduced suggesting that viraemia should also be considered in the repertoire of infectious complications with these iatrogenic neutropaenic patients. Reactivation of latent DNA viruses, for example, members of the *herpesviridae* family, and respiratory viruses have been reported in severely immunosuppressed patients after haematopoiesis stem cell transplantation (HSCT) but less so in patients that have been administered comparatively less aggressive chemotherapeutic treatment used against hematological malignancies. Since fever in these patients is sometimes the only indication of infection due to their immunosuppressed state, we not only determined the prevalence of viral infections in the iatrogenic neutropaenic adult patient we also related our findings to fever manifestation. Indeed, an association between virus detection and fever was observed, suggesting viral contribution to 30% of neutropaenic febrile episodes in our study cohort.

In addition to immune cell depletion, disruption of the gastrointestinal mucosa is another major side-effect since it can lead to microbial translocation from the large reservoir of bacterial microflora we harbour in our bowels. Elevated plasma endotoxins and sCD14 was observed in the bacteraemic episodes as well as in the episodes with fever of unknown origin (FUO). So, together with the 33% of the febrile episodes attributed to clinically documented bacteraemia and 30% attributed to viruses, we propose that aside from drug and tumour fever, a part of the remaining 37% of febrile neutropaenic episodes could be attributed to microbial translocation of bacterial products from the gut.

Acellular components of the immune system, such as the acute phase protein, mannose-binding lectin (MBL) have been suggested to be important in a similar cohort and that the use of replacement MBL therapy could be administered to reduce duration of febrile neutropaenic episodes. We however, did not observe any associations between MBL and infection type or frequency and add to the reports casting doubts on the benefit of recombinant therapy in the iatrogenic neutropaenic adult.

In conclusion, we have added to the panorama of infectious agents and bacterial products implicated during febrile neutropaenic episodes in the adult iatrogenic patient and have further discouraged suggestions for MBL replacement therapy.



## LIST OF PUBLICATIONS AND MANUSCRIPTS

- I. MICHELLE WONG, Lars Öhrmalm, Carl Aust, Martin Hibberd, Kristina Broliden and Thomas Tolfvenstam.  
**Mannose-binding lectin polymorphisms do not influence the frequency of infections in adults with neutropenia.**  
*PLoS ONE. 2012; 7(2):e30819. Epub 2012 Feb 17.*
- II. Lars Öhrmalm, MICHELLE WONG, Carl Aust, Per Ljungman, Oscar Norbeck, Kristina Broliden and Thomas Tolfvenstam.  
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**Flocked nasal swab versus Nasopharyngeal aspirate for detection of respiratory tract viruses in immunocompromised adults : a matched comparative study.**  
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- IV. MICHELLE WONG, Babilonia Barqosho, Lars Öhrmalm, Thomas Tolfvenstam and Piotr Nowak.  
**Microbial translocation contribute to febrile episodes in adults with chemotherapy –induced neutropaenia.**  
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## LIST OF ABBREVIATIONS

AdV	Adenovirus
ALL	Acute lymphoid leukaemia
AML	Acute myeloid leukaemia
ANC	Absolute neutrophil counts
BKV	BK polyomavirus
CAR	Coxsackie B and adenovirus receptor
CMV	Cytomegalovirus
CPE	Cytopathic effects
CRP	C-reactive protein
CRP	C-reactive protein
CRV	Community acquired Respiratory viruses
CT	Cycle threshold
DC	Dendritic cells
DTRF	Drug and treatment related fever
EBV	Epstein-Barr virus
EM	Electron microscopy
FluA	Influenzavirus A
FluB	Influenzavirus B
FN	Febrile neutropaenia
fNS	Flocked nasal swab
FUO	Fever of unknown origin
HCoV	Human coronavirus
HEV	Human enterovirus
HHV	Human herpesvirus
HMPV	Human metapneumovirus
HRV	Human rhinovirus
HSCT	Haematopoiesis stem cell transplantation
IFI	Invasive fungal infections
IL-	Interleukin -
JCV	JC polyomavirus
LBP	LPS-binding protein
LPS	Lipopolysaccharides
MBL	Mannose-binding lectin protein
<i>MBL2</i>	Mannose-binding lectin (protein C) 2
mCD14	Membrane-bound CD14
NHL	Non-hodgkins lymphoma
NPA	Nasal pharyngeal aspirate
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PCT	Procalcitonin
PIV 1-4	Parainfluenza virus 1-4
PML	Progressive multifocal leukoencephalopathy
PML	Progressive multifocal Leukoencephalopathy
qPCR	Quantitative PCR

RPMI 1640	Roswell Park Memorial Institute, media formula 1640
RS	Reed-Steinberg
RSV	Respiratory syncytial virus
RSV	Respiratory syncytial virus
sCD14	Soluble CD14
SNP	Single nucleotide polymorphism
TLR-	Toll-like receptors
TNF- $\alpha$	Tumour necrosis factor- alpha
VZV	Varicella-zoster virus



# 1 PREFACE

The human immune system is made up of a complex network of cells and molecules, each with an independent function yet, collaborating to achieve protection against invading pathogens we encounter daily and, developing a sustained memory to prevent infections when we next encounter the same pathogen again. It would be fair to say that ‘the whole is greater than the sum of the individual parts’ and a delicate balance is maintained between preventing infections and excessive tissue damage from pro-inflammatory responses.

Unfortunately, in situations where abnormal proliferation of malignant cells has occurred, the cytotoxic treatment strategy undertaken has an equally deleterious effect on cells of the immune system as well as the target malignant cells. Regardless, there is little recourse than to proceed with the treatment and deal with the consequences as they appear. A large proportion of which are infection related but the aetiology of the remaining is relatively unknown and suggested to be drug or tumour fever, in the absence of microbiological findings.

This thesis attempts to elucidate the aetiology of fever and infection susceptibility in the neutropaenic patient. In the first section of this thesis, an introduction to haematological disorders, infectious complications following cytotoxic chemotherapy, viral detection methods and immune alterations observed in this patient group is presented. This will lead on to a brief description of the materials and methods used, as well as, presentation of the main findings and concluding discussion of these results in relation to the triple goals of (1) discovering the contribution viruses to febrile neutropaenic episodes, (2) discovering the aetiology of febrile neutropaenic episodes with no attributable cause and, (3) ascertaining the role of an acute phase protein in infection susceptibility.

The intricate interactions involved in maintaining daily life as we know it is far more complex and it is not the intent of this thesis to tease it apart. A snapshot, describing observations in a particular time and space is perhaps a more apt depiction of the work presented here. A small, yet hopefully important, piece of the puzzle is presented for your deliberation.

Michelle Wong  
12 March 2012

## **2 INTRODUCTION**

### **2.1 THE IMMUNOCOMPROMISED HOST**

#### **2.1.1 Haematological malignancies**

Immune suppression can be attributed to many causes but will be limited to patients with haematological malignancies in this thesis. Common forms of haematological malignancies in adults can be broadly classified into leukaemias, lymphomas and multiple myelomas.

Leukaemias are a group of diseases exemplified by the neoplastic accumulations of malignant bone marrow precursor cells. Depending on the precursor cells, myeloblasts or lymphoblasts, the leukaemias are further classified into acute/chronic myeloid leukaemia or acute/chronic lymphoblastic leukaemia. Abnormal proliferation of these cells can lead to bone marrow failure by suppressing normal haematopoiesis which leads to anaemia, neutropaenia and thrombocytopaenia. Organ infiltration of these malignant cells can also occur if they gain entry into the circulatory system (Hoffbrand, 2006). In the adult patient, acute myeloid leukaemia (AML) is more common than acute lymphoid leukaemia (ALL) and the reverse is true for children.

Lymphomas are a highly heterogeneous group of disorders caused by the accumulation of mature lymphocytes in the lymph nodes, hence the classic clinical feature of lymphadenopathy. Occasionally, these malignant cells may enter the blood during the leukaemic phase or infiltrate other organs as well. Lymphomas can be further classified into two large groups based primarily on the presence of Reed-Steinberg (RS) cells. RS cells are found in Hodgkins disease and all other lymphomas without RS cells are termed 'Non-Hodgkins Lymphoma' (NHL). RS cells observed in Hodgkins disease, which account for less than 10% of all lymphomas, has been suggested to originate from B-cells with mutations which prevent synthesis of full length immunoglobulins (Hoffbrand, 2006). Non-Hodgkins lymphoma (NHL), on the other hand, describes a larger, less well-defined and more variable diseases. Classification of diseases in NHL is inherently tricky but consensus achieved with the Revised European-American Lymphomas classification (REAL) (Harris et al., 1994) and World

Health Organisation (WHO) (Vardiman et al., 2009) has helped with subdividing lymphomas based on immunophenotyping, cytogenetic and molecular characteristics.

Multiple myeloma is a disease caused by neoplastic proliferation of bone marrow clonal plasma cells and is additionally characterised by lytic bone lesions and the presence of monoclonal immunoglobulins in serum and urine. Located throughout the bone marrow, clumps of malignant plasma cells appear and thus the term 'Multiple myeloma'. Primarily an adult disease, the aetiology is relatively unknown although cytokines, for example IL-6 has been suggested to be a potent growth factor for multiple myelomas (Hoffbrand, 2006).

### **2.1.2 General treatment strategies**

Targeting abnormal and prolific neoplasm, treatment strategies are generally curative and directed towards limiting the further growth and spread of the malignancy. Most chemotherapeutic drugs fall into a few main categories; alkylating agents, antimetabolites, plant alkaloids, topoisomerase inhibitors and 'antitumour antibiotics'.

Alkylating agents act by attaching an alkyl group to a DNA base, usually the guanine base, leading to cross-linking of the DNA double-helix strands thus preventing further cell division. Anti-metabolites are generally nucleoside analogues (purine or pyrimidine) that inhibit cell division and RNA synthesis by competing with intrinsic purines and pyrimidines. Alkaloids derived from plants prevent normal microtubule function hence, halting cell division while topoisomerase inhibitors interfere with both transcription and replication of DNA by disrupting DNA unwinding and supercoiling. Lastly, 'antitumour antibiotics', so called because of their initial discovery in microorganisms, include a wide range of compounds for example, anthracyclines and DNA intercalators (Airley, 2009). Monoclonal antibodies specifically targeting lysis of CD20+ B- lymphocytes has been used and is an approach away from generic inhibition of cell proliferation.

Corticosteroids are sometimes included in combination to suppress potential inflammation and has been reported to induce lymphocyte apoptosis (Zitvogel et al., 2008) and dendritic cell (DC) dysfunctions. Regardless of the drugs used, all of them exert their effect by interfering with cell division and DNA synthesis and these effects are seldom specific to the malignant cells only. Bystander damage to other normally

rapidly dividing cells, for example, cells of the immune system and gut epithelial cells often occur, leading to further infectious complications which will be described in the ensuing chapters.

### **2.1.3 Febrile neutropaenia in the iatrogenic immunosuppressed patient**

Neutropaenia is a common occurrence as a result of the cytotoxic chemotherapeutic treatment and is associated with increased risk of infections (Bodey et al., 1966). Variably defined, neutropaenia is said to have occurred when the absolute neutrophil count drops below  $1 \times 10^9$  cells/ml of blood. While an immunocompetent individual is able to present with a myriad of symptoms upon infection, with the immunosuppressed patient, fever is often the primary and only clinical sign of an infection. Infectious complications can potentially have fatal consequences hence an occurrence of infection-related fever prompts empiric antimicrobial therapy and necessitates delays and disruptions in the chemotherapeutic treatment for the underlying haematological malignancy (Pizzo, 1999). However, in 30%-55% of febrile neutropaenic episodes, no attributable cause for the fever can be ascertained using routine microbiological diagnostics and these are termed fever of unknown origin (FUO) (Hahn-Ast et al., 2010, Pagano et al., 2011b, Viscoli et al., 2006). Drug and tumour fever or, necrotic cell death as a result of the cytotoxic treatment could trigger fever just as well as infection thus the non-specificity of fever renders it an unreliable diagnostic marker for infection in this patient group. The severity of infections in these patients has prompted studies evaluating biomarkers able to distinguish infection-related fevers from otherwise 'sterile' fevers. C-reactive protein (CRP) (Koivula et al., 2011, Manian, 2012, Sakr et al., 2008), pentraxin 3 (Juutilainen et al., 2011) and procalcitonin (PCT) (Koivula and Juutilainen, 2011, Massaro et al., 2007, Ruokonen et al., 1999, Sakr et al., 2008, Schuttrumpf et al., 2003, Simon et al., 2004) have been studied for this purpose, but their efficacy in identifying bacteraemia induced fevers is still not convincing.

## **2.2 INFECTIOUS COMPLICATIONS**

Infections are common in the immunocompromised host (Pizzo, 1999, Menichetti, 2010) in particular, patients that have undergone chemotherapy are at higher risk due to the disruption of mucosa (Duncan and Grant, 2003, Lehrnbecher et al., 1997, Meckler

and Lindemulder, 2009) and the use of indwelling devices like central venous and urinary catheter (Meckler and Lindemulder, 2009). With more intensive treatment for haematological malignancies, the number of immunocompromised patients will undoubtedly increase. Infections in the neutropaenic patients account for 50%-60% (Pagano et al., 2011b) of fever episodes and if left untreated, could lead to sepsis and be potentially fatal (Table 2.2-1). Pre-emptive antimicrobial therapy (Maschmeyer et al., 2003, Wingard, 2004) has thus been the strategy taken but the emergence of antibiotic resistant bacterial strains has prompted many clinicians to exercise caution on the prolific use of these drugs (Akova et al., 2005, Krcmery and Beno, 2005). By virtue of their neutropaenia, bacterial and fungal infections are most common, with viral infections only observed in low proportions of febrile episodes (Norgaard et al., 2006, Pagano et al., 2011a). The skewed observation could also be a reflection of the microbiological focus in earlier investigations. However, in this thesis, focus will be placed on viral infections with short discussions on bacteraemia and invasive fungal infections for an overall perspective of infectious complications in the immunocompromised host.

**Table 2.2-1.** List of common infectious agents in the immunosuppressed host (Meckler and Lindemulder, 2009, Menichetti, 2010)

Bacterial		Viral		Fungal
<u>Gram-Positive</u>	<u>Gram-Negative</u>	<u>Blood-borne</u>	<u>Respiratory</u>	
Staphylococcus spp.	Escherichia coli	Cytomegalovirus	Respiratory Syncytial virus	Candida spp.
Streptococcus spp.	Pseudomonas aeruginosa	Epstein-Barr virus	Influenzavirus A and B	Aspergillus spp.
Enterococcus spp.	Klebsiella spp.	BK virus	Parainfluenza virus	Zygomycetes
Corynebacterium spp.	Enterobacter spp.	Herpes simplex virus	Adenovirus	Fusarium spp.
Bacillus spp.	Anaerobes	Varicella-zoster virus		Scedosporium spp.
Clostridium spp.		Enterovirus		
		Rotavirus		
		JC virus		
		Parvovirus B19		

### 2.2.1 Bacteraemia

Bacteria, as expected, represent the majority of identified pathogen in infectious complications faced by the neutropaenic host. However, the proportions of Gram-negative to Gram-positive bacteraemia have fluctuated over the decades (Ellis, 2008). Before the 1980s, Gram-negative bacteraemia especially species originating from the gastrointestinal tract typified by, *E.coli*, *Kleibsell*a spp. and *Pseudomonas* spp. predominate. The introduction of beta-lactam antibiotics led to diminishing Gram-negative bacteria numbers and shifted the balance towards one where Gram-positive bacteraemia dominated. *Staphylococcus* spp., coagulase-negative *Streptococcus* spp. and *Enterococcus* spp. and more alarmingly, *Streptococcus viridians* were increasingly seen and have remained so till recently where *Pseudomonas aeruginosa* and *Enterobacteriaceae* have been increasingly documented (Cometta et al., 1996, Cometta et al., 2003, Kern et al., 1999, Klastersky et al., 2007, Mancini et al., 2008, Menichetti, 2010, Viscoli, 2002, Viscoli et al., 2005, Wisplinghoff et al., 2003).

The use of prophylactic antibiotics as pre-emptive treatment for the immunocompromised host has undoubtedly reduced bacteria-associated mortality. However, antibiotic resistant *Strep. viridians*, *Enterococci* spp. and *E.coli* have emerged due to excessive use of beta-lactam antibiotics and quinolones thus, judicious use of antibiotics is encouraged.

### 2.2.2 Invasive fungal infections (IFI)

Invasive fungal infections have been documented in 2%-40% of immunosuppressed patients with haematological malignancies and prolonged neutropaenia after chemotherapy (Bohme et al., 2009, Cuenca-Estrella et al., 2008, Hahn-Ast et al., 2010, Marr et al., 2002b, Martino and Subira, 2002, Muhlemann et al., 2005, Pagano et al., 2006, Whimbey et al., 1987). *Candida* spp. and *Aspergillus* spp. are most commonly implicated in invasive fungal infections but the proportions have varied through the years depending on the use of azole prophylaxis (Akova et al., 2005, Ellis, 2008, Hahn-Ast et al., 2010, Krcmery and Beno, 2005, Marr, 2000, Marr et al., 2002a, Marr et al., 2002b, Marr et al., 2000, Menichetti, 2010, Pagano et al., 2011a). However, reduced susceptibility to azoles (ketoconazol, fluconazol and itraconazol) in non-albicans *Candida* spp. has been observed in some centres and is a growing concern (Krcmery

and Babela, 2002). The emergence of *Zygomycetales*, resistant to the latest antimicrobial treatments is also worrying.

### **2.2.3 Viraemia**

Viral infections that are often self-limiting in the healthy individual can represent a larger threat to the immunocompromised host. In the course of our lives, we would have encountered and overcome a multitude of viral infections but compromising the immune system, especially the T-lymphocytes targeting these virus places the immunocompromised host at great risk (Christensen et al., 2005, Hakim et al., 2009, Wade, 2006).

#### *2.2.3.1 Reactivation of latent viruses*

##### Herpesviruses

Human Herpesviruses are members of the Family *Herpesviridae* and are linear dsDNA viruses and range in size from 125nm to 260nm. The icosahedral capsid is enveloped with viral glycoprotein spikes on the surface and the variation in sizes is suggested to be due to variations in the tegument layer in between.

**Table 2.2-2.** List of human herpesviruses commonly associated with the immunosuppressed host. (Adapted from (Philip E. Pellet, 2007)

Designation	Name	Genome size (kbp)
Human herpesvirus 1 (HHV-1)	Herpes Simplex virus 1	152
Human herpesvirus 2 (HHV-2)	Herpes Simplex virus 2	155
Human herpesvirus 3 (HHV-3)	Varicella-zoster virus	125
Human herpesvirus 4 (HHV-4)	Epstein-Barr virus	172
Human herpesvirus 5 (HHV-5)	Cytomegalovirus	230

All human herpesviruses have the ability to remain latent in their natural hosts, taking on the form of closed circular molecules with the expression of only a small subset of genes during latency (Pellet and Roizman, 2007). The mechanism of reactivation from latency varies between the virus species but in the immunosuppressed host, the altered immune system especially depletion of cellular immunity, is the most likely explanation.

Herpes simplex virus reactivation can manifest as ulcers in the mucocutaneous zones and can be difficult to distinguish from chemotherapy-related mucositis (Hakim and Shenep, 2010). Varicella-zoster (VZV) infections in adults with haematological malignancies are reactivated from earlier infections (Wade, 2006, Locksley et al., 1985) while VZV naïve patients should be wary of contracting the infection from the community and vaccination of either themselves or close familial contacts is advised. Epstein-Barr virus residing in B-lymphocytes can proliferate in the absence of T-lymphocyte control and can lead to post-transplant lymphoproliferative disease (PTLD) (Rickinson and Kieff, 2007). Last, and certainly not least, cytomegalovirus reactivations are significant in the immunocompromised host with impaired T-lymphocyte immunity and account for substantial morbidity and mortality in these patients (Hakim and Shenep, 2010). The disease manifests as pneumonitis, enterocolitis, hepatitis, encephalitis, cystitis, nephritis, bone marrow suppression and



multi organ system failure (Boeckh et al., 2003, Hakim and Shenep, 2010, Hebart and Einsele, 2004, Nguyen et al., 2002, Wade, 2006). Of importance in patients immunosuppressed for HSCT procedures, the relevance of herpesviruses in chemotherapy-induced neutropaenic patients has not been clearly established.

### Adenovirus

Human Adenoviruses are dsDNA (26-45kbp), non-enveloped viruses of the family *adenoviridae*. They are medium sized viruses, approximately 90nm in diameter, with fibres projecting from the vertices of the icosahedral capsid. Since its discovery in 1953, more than 51 human adenovirus serotypes have been identified, based on their resistance to neutralisation by antisera to other known human adenovirus (De Jong et al., 1999, Hierholzer et al., 1991). These serotypes can be further categorised into seven serogroups (A-G) based on their ability to agglutinate red blood cells (ICTV, 2009 ).

Aside from Group B adenoviruses, all other adenoviruses bind to the plasma membrane Coxsackie B virus and adenovirus receptor (CAR). Ubiquitous expression of CAR in epithelial cells of various tissue types, gastrointestinal tract, respiratory tract, explains the variation of symptoms observed with adenovirus infection. Human adenovirus has been associated to a variety of clinical syndromes, upper and lower respiratory disease, conjunctivitis, gastroenteritis, haemorrhagic cystitis, hepatitis, to name a few. In immunocompetent patients, adenovirus usually causes a mild self-limiting acute infection but have the ability to persist in the adenoid vegetations of the nasopharynx and in tonsils and, B lymphocytes have been observed to harbor viral DNA in a non-productive state (Horvath et al., 1986, Neumann et al., 1987, Rajcani, 2007). Secretion of subgroup c adenovirus into faeces long after resolution of the initial acute upper respiratory tract infection (Adrian et al., 1988, Fox et al., 1969, Fox et al., 1977) and the recovery of Adv3 from the tears of patients, 10 years after acute conjunctivitis (Kaye et al., 2005) are further evidence for the persistence of AdV.

In post-transplantation immunosuppressed patients, adenovirus can cause fatal infections resulting in hepatitis abnormalities, pneumonia and encephalitis (Bowles and Vallejo, 2003, Krilov, 2005, Wold and Horwitz, 2007). Interestingly, adenovirus strains identified in the immunocompromised patient were no different from ubiquitous serotypes prevalent in patients with other, less serious illnesses suggesting that the

immunocompromised host may be no more commonly infected, rather that the outcome of the infection more severe (Wold and Horwitz, 2007). In liver transplant patients, reactivation of latent adenovirus in the donor organ as a source of adenovirus infection has also been documented (Cames et al., 1992). Deaths from adenoviraemia is closely linked to recovery of T-lymphocytes function following immunosuppression (Chakrabarti et al., 2002, Heemskerk et al., 2005, Kampmann et al., 2005, van Tol et al., 2005) and to some extent adenoviral loads in peripheral blood (Claas et al., 2005). The relevance of adenoviral infections in the non-transplant immunosuppressed patient is less studied.

#### 2.2.3.2 *Opportunistic viral infections*

##### Parvovirus B19

Parvovirus B19 was the first known human virus in the *parvoviridae* family. It is a small (18-26nm), non-enveloped virus with a linear ssDNA encapsulated in an icosahedral capsid. Human transmission of the virus is suggested to be via aerosol inhalation, faecal-oral route and via infected blood products. Target cells for parvovirus B19 are the erythroid progenitor cells, through the P antigen receptor, located in the bone marrow although, occasionally the virus infects leukocytes (especially neutrophils) (Mustafa and McClain, 1996).

Viremia is generally an acute, bi-phasic event (Anderson et al., 1985), with non-specific influenza-like symptoms, including fever, malaise and myalgia (Burns and Parrish, 2007) and erythrocyte aplasia. Acute infection with parvovirus B19 causes the childhood erythema infectiosum (fifth's disease). Peak occurrence of parvovirus B19 infections are seasonal and occur in late winter, spring and summer (Young, 1995) and are often spread among schoolchildren and sibling-sibling transmission. Upon clearance of the acute infection, individuals develop long-lasting antibody-mediated immunity (Srivastava et al., 1990).

Immunocompromised patients as a result of chemotherapy or immunosuppressive drugs may develop chronic parvovirus B19 infection, resulting in prolonged cytopenia, especially anaemia that interferes with their planned course of chemotherapy (El-Mahallawy et al., 2004, Lindblom et al., 2008, Soliman Oel et al., 2009).

### Polyomavirus

The two human polyomaviruses, JC virus (JCV) and BK virus (BKV) were originally isolated from immunocompromised hosts in 1971 (Gardner et al., 1971, Padgett et al., 1971) and their relevance in this patient group remains although the mechanism of action may still be unclear. Polyomaviruses are non-enveloped particles 40-45nm in diameter, with a covalently closed circular dsDNA approximately 5kbp long.

Cell receptors for BKV and JCV are not completely established but BKV has exhibited tropism towards gangliosides and N-linked glycoproteins containing sialic acid moieties (Dugan et al., 2005, Low et al., 2006, Seganti et al., 1981). JCV entry into the cell is however suggested to be via clathrin-mediated endocytosis after binding to  $\alpha(2,6)$ -sialic acid and 5HT<sub>2A</sub> serotonin receptor (Elphick et al., 2004). These two viruses are ubiquitous in most populated populations, as evidenced by seroepidemiology studies (Knowles et al., 2003). Antibodies remain throughout life but can fluctuate in titre although, it is unknown if it is a result of reinfection or reactivation infections. Maintenance of chronic viral infection is another biological characteristic of JCV and BKV, with viral shedding in the urine from a chronically infected kidney. Aside from urine and the primary organ of infection, both viruses have been detected in peripheral blood (Azzi et al., 1996) and in some studies with the American and European populations, it has been estimated that 2% to 3% of the with no underlying disease carry JCV DNA (Dubois et al., 1997).

BKV-associated haemorrhagic cystitis (Leung et al., 2001, Silva Lde et al., 2010) and progressive multifocal leukoencephalopathy (PML) as a result of JCV reactivation are potentially severe complications in the immunocompromised host especially patients on immunosuppressive drugs for graft protection. Regardless of the virus species, either reactivation, chronic or opportunistic infection of polyomavirus has an impact on the immunocompromised host as it might delay chemotherapy treatment for their underlying disease or necessitate reduction of immunosuppressive drugs required for engraftment post-transplant.

### Respiratory virus infections

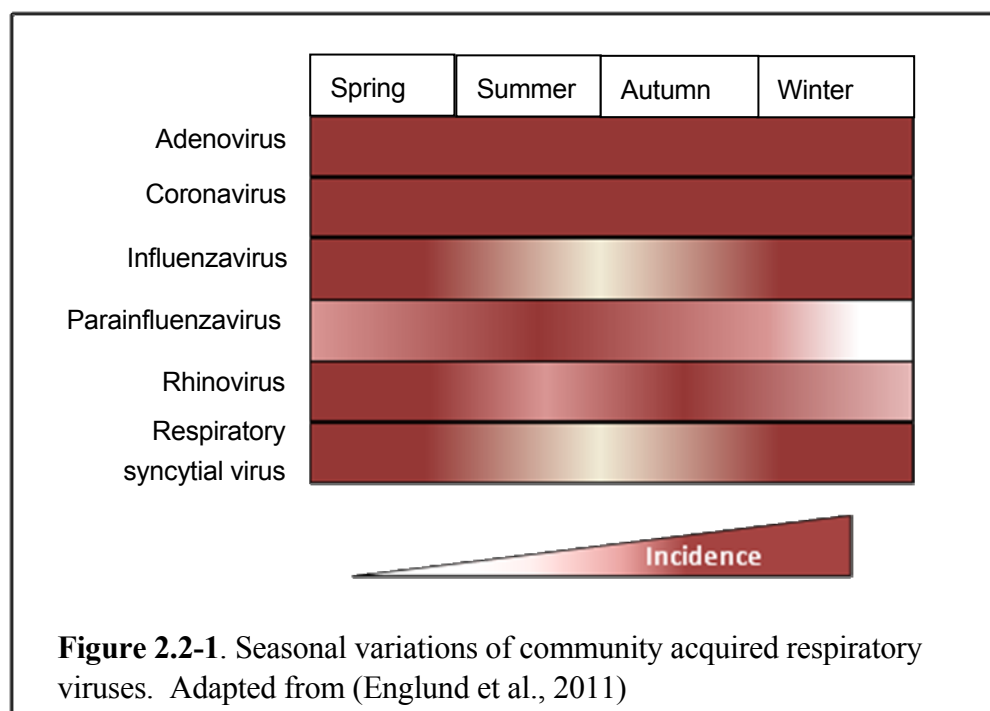
Respiratory viral infections are widespread in the community and are therefore easily spread to immunosuppressed patients with underlying haematological malignancies.

General characteristics of viruses commonly implicated in respiratory infections are listed in table 2.2-3. The term community acquired respiratory virus (CRV) infections is apt as the patient generally acquires the disease from family members or medical staff in the hospital (Bowden, 1997, Couch et al., 1997, Harrington et al., 1992, Ljungman, 1997, Nichols et al., 2001, Shah and Chemaly, 2011, Whimbey et al., 1997, Englund et al., 2011, Englund et al., 1999, Hakim and Shenep, 2010, Wade, 2006). Influenzaviruses (A and B), respiratory syncytial virus, parainfluenzaviruses (1-2), adenoviruses and, possibly rhinoviruses, are the most common CRVs (Bowden, 1997, Couch et al., 1997).

**Table 2.2-3.** General characteristics of respiratory viruses

Family	Virus species	Genome	Genome size	Envelope	Host receptors
<i>Picornaviridae</i>	Human rhinovirus	(+) RNA	~7kb	No	ICAM-1 or LDL
<i>Paramyxoviridae</i>	Respiratory syncytial virus	(-) RNA	~15kb	Yes	Glycosaminoglycan
<i>Paramyxoviridae</i>	Parainfluenza virus 1-4	(-) RNA	~15kb	Yes	Sialic acid
<i>Paramyxoviridae</i>	Human meta-pneumovirus	(-) RNA	~13kb	Yes	unknown
<i>Orthomyxoviridae</i>	Influenzavirus A and B	Segment, (-)RNA	~15kb	Yes	Sialic acid
<i>Coronaviridae</i>	Human coronavirus	(+) RNA	~30kb	Yes	Aminopeptidase N, 9-o-acetylated sialic moieties or angiotensin-converting enzyme 2.
<i>Adenoviridae</i>	Human adenovirus	Linear dsDNA	~36kbp	No	CAR
<i>Parvoviridae</i>	Bocavirus	Linear ssDNA	~6kb	No	Unknown

Human metapneumoviruses and bocaviruses have also been recently identified in symptomatic transplant recipients (Boivin et al., 2002, Koskenvuo et al., 2008, Peret et al., 2002, Schenk et al., 2007) although their relevance is unclear. In temperate countries, the virus species responsible for respiratory infections vary throughout the year (Fig. 2.2-1) and the seasonality of CRV infections in the community is reflected in the immunocompromised host as well.



An immunocompetent individual usually clears the infection with mild symptoms whilst prolonged infection with RSV (Craft et al., 1979, Fishaut et al., 1980, Hall et al., 1986, Harrington et al., 1992, King et al., 1993, McIntosh et al., 1984), parainfluenza-3 (Fishaut et al., 1980, Gelfand et al., 1983, Josephs et al., 1988) and influenza A (Craft et al., 1979) occurs in the immunocompromised patient (Couch et al., 1997). In 50%-70% of immunocompromised patients (bone-marrow transplant or induction chemotherapy for leukaemia) with CRV develop pneumonia, with mortality ranging from 9% to 45% (Bowden, 1997, Couch et al., 1997, Harrington et al., 1992, Ljungman, 1997, Whimbey et al., 1997). Thus, prompt identification of the respiratory viral pathogen is critical for the initiation of specific antiviral therapy or even, the potential delay of immunosuppressive therapy or transplantation to avert the severe consequences of CRV infections.

Many other viruses have been associated with the immunocompromised host, enterocolitis from rotavirus infection (Stelzmueller et al., 2007) and gastroenteritis from norovirus infection in HSCT recipients (Roddie et al., 2009, Saif et al., 2011). The degree and duration of immunosuppression are major risk factors for reactivation of latent viral infections and opportunistic viral infections.

#### **2.2.4 Polymicrobial infections**

In 8% to 25% of blood bacteraemic infections, polymicrobial infections are documented in treatment-induced immunosuppression (Gupta et al., 2010, Norgaard et al., 2006, Rolston et al., 2007, Cometta et al., 1996, Del Favero et al., 2001, Giamarellou et al., 2000, Gonzalez-Barca et al., 1999, Jacobson et al., 1999, Klastersky et al., 2007, Peacock et al., 2002, Rossini et al., 2005, Sanz et al., 2002, Sigurdardottir et al., 2005, Whimbey et al., 1987). These could be co-infections with more than one species of bacteria or, co-infections of bacteria with fungal or viral infections (Cometta et al., 1996, Del Favero et al., 2001, Giamarellou et al., 2000, Gonzalez-Barca et al., 1999, Jacobson et al., 1999, Klastersky et al., 2007, Peacock et al., 2002, Rossini et al., 2005, Sanz et al., 2002, Sigurdardottir et al., 2005, Whimbey et al., 1987). Although the definition of polymicrobial infections is not standardised, it is apparent through the years that polymicrobial infections do occur. The relevance, other than indicating a more severe immunosuppression, is however, debatable. Regardless, a better estimation of the microbial load in this vulnerable group could enable a better informed treatment of infections.

#### **2.2.5 Microbial translocation**

A large population of bacteria, in the order of  $10^{12}$ /mm, resides within the estimated 200 m<sup>2</sup> human intestinal lumen surface (Ellis, 2004, Balzan et al., 2007) yet an immunocompetent individual is relatively disease-free due to an intact mucosal immunological defence. Disruption of the gut mucosa, particularly the enterocytes, as a result of cytotoxic treatments renders the immunocompromised host at risk for the translocation of bacteria and bacteria-products into the circulatory system.

The two major routes of bacterial translocation are (1) transcellular, through the enterocytes and (2) paracellular, using the tight junctions. (Balzan et al., 2007, Ellis, 2004). Structural damage to enterocytes as a result of cytotoxic chemotherapy suggests

that the paracellular route of translocation predominates in patients undergoing treatment for haematological malignancies (Gasbarrini and Montalto, 1999, Keefe et al., 2000). Thereafter, the principal pathway for entry into systemic circulation is via the lymphatic enteric drainage (Deitch, 2002), followed by draining via the intestinal subepithelial capillaries (Balzan et al., 2007, Moore et al., 1991).

Lipopolysaccharides (LPS) and some glycopeptides found on surfaces of commensal intestinal bacteria are pyrogens and potent activators of pro-inflammatory response via Toll-like receptor 4/MDA-2 complex (Poltorak et al., 1998). LPS in itself, even in the absence of live bacteria is sufficient to initiate the pro-inflammatory cascade and cause fever. The binding receptor of the LPS monomer is sCD14, which activates pro-inflammatory response by presenting LPS monomers to TLR4 directly or via mCD14 (Hailman et al., 1996) and initiating the release of cytokines such as Tumour necrosis factor-  $\alpha$  (TNF- $\alpha$ ) and interleukins 1 and 6 (IL-1, IL-6). sCD14 however, has an alternate role of inhibiting excessive pro-inflammatory cytokine release by shuttling LPS to circulating lipoproteins, where they eventually cleared. The same can be said of the LPS-binding protein (LBP) which alternately acts as a catalyst accelerating LPS binding to sCD14 (Jack et al., 1997, Yang et al., 2002, Knapp et al., 2003, Fan et al., 2002) as well as promoting the clearance of LPS via binding to lipoproteins (Wurfel et al., 1994, Kitchens et al., 2003). The dual bioactivity roles of sCD14 and LBP ensure that effective clearance of bacterial pathogens at the local site of infection is not achieved at the expense of unnecessary tissue damage through excessive stimulation of circulatory pro-inflammatory cytokines.

The presence of flagellin, another bacterial product is also capable of initiating a pro-inflammatory cascade via Toll-like receptor 5. Thus, in the absence of bacteraemia, patients with microbial translocation may still develop febrile symptoms, indicative of an infection. Some early studies had suggested that translocation of microbial products might explain fever in chemotherapy-immunosuppressed patients (Gunther et al., 1995, Harris et al., 1984, McCartney et al., 1987, Tancrede and Andremont, 1985) although their findings are in some cases limited by their use of faecal bacterial cultures as indicators for microbial translocation. The degree of mucosal disruption would determine if it would be the whole bacteria or simply bacterial products that would translocate and the use of faecal bacterial cultures will not be able to reflect the latter

situation. Assays targeting the LPS systemically, together with the faecal cultures would allow better estimation.

## **2.3 VIRAL DETECTION METHODS IN IMMUNOSUPPRESSED PATIENTS**

Viral detection methods have developed much over the years and there are often more than one method that can be used for any viral species, but the focus here will be the relevance in the immunocompromised patient. In general, viral detection methods fall into one of the following categories: (1) Virus culture and direct visualisation; (2) Viral antigenic detection; (3) Serology and; (4) Nucleic acid detection. Although discussed separately, virus culture and visualisation is often coupled with viral antigenic detection for improved specificity.

### **2.3.1 Virus culture and direct visualisation**

The use of virus culture for detection has limited use despite its standing as a ‘gold standard’. In general, cytopathic effects (CPE) in the cell monolayer are used to indicate presence of viruses but this is rather non-specific. Although well controlled in the laboratory setting, cultured cells die off from a multitude of reasons and can sometimes be mistaken for CPE. Virus tropism is another major flaw in the virus culture system which means that a large array of immortalised cell lines needs to be maintained. The use of primary cells, able to support a wider range of viruses is a possibility if the cost of maintaining fresh cultures can be overcome.

Histological investigations with light microscopy, used in conjunction with virus culture can be useful in the detection of some viruses, for example with rabies virus where viral inclusion bodies, termed negri bodies are observed in the cytoplasm of infected nerve cells. However, such specific cellular changes are not always present in many viral infections and the use of electron microscopy for direct visualisation of the virus is preferred.

Electron microscopy (EM) has long been the hallmark of virus detection methods. The large magnifications achievable by EM afford the observer an opportunity to actually view the virus particle, which would otherwise be an abstract object. To a well trained



operator, virus identification can be performed directly from clinical samples. However, the major limitation of EM is the lack of sensitivity with typically,  $10^5$  to  $10^6$  virus particles/mL are required for reliable visualisation. Virus culture prior to EM visualisation is a way to circumvent the limitation in sensitivity but suffers from the drawbacks as mentioned above. Even when an image of the virus is captured, the information gleaned would be restricted in the presence of commensal viral populations for example in gastrointestinal tract or an immunosuppressed patient highly susceptible to a multitude of viral infections. Nonetheless, EM has been and still is, extensively used to investigate virus structures and in situations where speed and sensitivity is not crucial.

### **2.3.2 Viral antigenic detection**

Antigens present on viruses are the targets of these methods which improve on the limited specificity of direct visualisation methods. The most commonly practiced technique is immunofluorescence coupled with microscopy. Antibodies specific to viral antigens are fluorescently labeled so that one may visualise the location of the virus particle within the cell. Evidently, the specificity of the assay is highly dependent on the choice of antibodies directed towards the viral antigen. Although the technique has the ability to improve specificity, sensitivity is still limited.

### **2.3.3 Serology**

The bulk of virus detection methods are serology based, detecting antibodies developed upon exposure to viruses. These include classical methods, complement fixation tests (CFT), Haemagglutination inhibition assays (HIA) and neutralization tests as well as, 'newer' techniques for example, western blots, and enzyme linked immunosorbent assays (EIA). A natural primary humoral response occurs when we encounter viral antigens and this is generally specific for the viral species. Cross-reactivity to viral antigens does of course occur for example with some of the flaviviruses. The target molecules in serological assays are usually the IgM or IgG based on the principle that IgM titres rise rapidly in an acute infection, followed by an increase in IgG after a couple of weeks but as in all things biology, there are always exceptions to the rule. With respiratory viral infections, many viruses produce clinical disease before the appearance of antibodies. Another inherent difficulty with these methods is in defining a threshold which constitutes a rise from background. This is commonly determined by comparing antibody levels from samples taken during the acute phase with another

taken during the convalescent phase. Aside from epidemic situations, there is limited utility in identifying the causative virus after cessation of the course of infection in an individual patient. An immunocompromised patient, especially one with impaired B-lymphocyte cells and function, is unlikely to produce a strong enough humoral response for reliable detection and transfusions may blur IgG boosting.

#### **2.3.4 Molecular detection**

Molecular methods for viral detection are the latest methods in the toolbox of the virologist. Nucleic acid based detection methods are growing in popularity as it offers improved specificity and sensitivity. The dropping costs in the running of these assays in recent years have made these methods a highly attractive option. Today, nucleic acid detection methods are almost synonymous with PCR but it is important to remember other methods which can be used too, Southern blots, Northern blots and Nucleic acid based amplification (NASBA). Taking the basic ideas forward, microarrays capable of screening hundreds of viruses have been developed (Wang et al., 2002) and sequencing is becoming a viable option for virus detection in view of the rapid development of deep sequencing instrumentation (Beerenwinkel and Zagordi, 2011).

PCR is a highly specific and sensitive tool suitable for use in the detection of viruses. The rapid turnaround from sampling to result is extremely attractive for clinical applications where treatment modalities can be tailored. PCR sensitivity is based on the highly efficient amplification of target sequences with a DNA polymerase, Taq polymerase while its specificity is dependent on the design of primers that selectively bind to the target sequence. Used together with a fluorescent probe, additional quantitation ability and specificity is conferred but more importantly, amplification of the target sequence can be monitored in real-time as a result of the fluorescent signal emitted from the cleaved probe. Together with improvements in equipment, PCR offers specific, sensitive and rapid results.

The sensitivity of PCR is however, both its blessing and its curse. Theoretically capable of detecting single copies of viral nucleic acids, it offers results beyond any of the other methods can but the relevance of finding minute quantities of viruses which may or may not be viable is debatable. The dogma that some body fluids, for example cerebrospinal fluids, should be sterile has been used to justify the definition of viral infection when virus nucleic acid is detected, and rightly so. This assumption does not

holdup in respiratory secretions where aerosolised viral particles may be encountered, or in blood samples of patients harbouring latent viral infections. In theory, a viral infection is said to have occurred if even a single cell is infected but this does not constitute a symptomatic disease and better associations with viral nucleic acid copy numbers are required. PCR contamination, as a result of remnant DNA products or simply from the sampling procedure would also be amplified along with the target virus and could also result in misleading information.

Microarrays based on the hybridisation with probes immobilised on a slide, build on the specificity of PCR and enable high throughput screening against large panels of virus species. Prior knowledge of viral genome sequences is necessary for the design of good probes and is also the intrinsic flaw of the technique and one is limited by the current sequence database. Next generation deep sequencing platforms attempt to address this flaw with the promise of unbiased high throughput sequencing, enabling detection of true unknown viruses. The primary strength of deep sequencing lies in the increased coverage of each target sequence but more importantly, the ability to sequence unanticipated sequences on account of the unbiased sequencing reaction. In this way, sequence readouts are theoretically possible for every available nucleic acid sequence in the sample. This is thus potentially the method of choice for the detection of true unknown viruses. Of course, in the presence of an overwhelming amount of sequence data there is still a possibility of dismissing unusual sequences, and herein, the age-old requirement of experience might help, or not.

In the immunosuppressed patient, molecular detection of viruses might be the most cost-effective option since serological assays are less useful due to their immunosuppression and frequent transfusions.

## **2.4 IMMUNE ALTERATIONS IN THE IMMUNOSUPPRESSED HOST**

Despite encountering microorganisms daily in our lives, we do not fall ill more frequently and the immune system has a large part to play in our defence against microbial diseases. The immune system however, is made up of many components, each working individually but still in cohort with each other. The complexity of interactions is maintained in an inter-dependent balance, the disruption, for example in

the case of the immunosuppressed host, invariably increases susceptibility to infections. In general, the immune system can be divided into two arms; (1) the adaptive immune system and (2) the innate immune system. They are so named to reflect the typical roles they play. Cytotoxic chemotherapy treatment is necessary for the treatment of the underlying haematological malignancy but naturally also depletes the cells required in the immune system (Lehrnbecher et al., 1997). This effect will be discussed in further detail in the following sections.

## **2.4.1 Adaptive immune system**

### *2.4.1.1 T-lymphocytes*

Chemotherapy dose regimen administered for the treatment of the underlying haematological malignancy naturally plays a central role in degree of T-cell immunosuppression and the rate of T-cell recovery (Boldt et al., 1984, Cheson, 1995, Hersh et al., 1971, Kraut et al., 1990, Layward et al., 1981, Strender et al., 1981, Urban et al., 1989, Weiner and Cohen, 2002, Zitvogel et al., 2008). Subtle difference however exist in the manner the two T-cell subsets (CD4+ and CD8+) respond to the cytotoxic agents. CD4+ T-cells are not only depleted to a larger extent, recovery is also slower than those of CD8+ T-cells (Mackall et al., 1997, Mackall et al., 1994). Further, fewer regenerated CD4+ T-cells are of the 'naïve' CD4+ phenotype but rather predominantly bear signs of activation and belong to the 'memory' CD4+ phenotype (Haining et al., 2005, Mackall et al., 1994). Age is inversely correlated to the proportions of 'naïve' phenotype CD4+ T-cells regenerated and this presumably reflects diminished thymic activity with age (Borella et al., 1972, Mackall et al., 1994, Storek et al., 1995, Weinberg and Parkman, 1995) although the rapid regeneration of CD8+ T-cells suggest that a thymic-independent pathway for T-cell regeneration might also play a role. Regardless, even if T-cell numbers are recovered, they are of a more restricted repertoire. Dysfunction of regenerated T-cells have also been reported (Borzy and Ridgway, 1989, Chung et al., 1977, Katz et al., 1987, Layward et al., 1981, Magrath and Simon, 1976) and it is clear that these cytotoxic drugs not only deplete the existing T-cells, a profound effect on the change in their phenotype and function occurs as well.

#### 2.4.1.2 Immunoglobulins

Sub-optimal or absent immunoglobulins could predispose the immunosuppressed patient to infection from encapsulated bacteria, especially *Streptococcus pneumoniae* and has prompted the introduction of replacement therapy with commercial immunoglobulins (Abrahamsson et al., 1995, Alanko et al., 1992, Borella et al., 1972, Hitzig et al., 1976b, Hitzig et al., 1976a, Katz et al., 1987, Reid et al., 1977). Patients undergoing haematopoietic stem cell transplantation (HSCT) are particularly at risk due to the intensity of their cytotoxic treatment. Following HSCT, mature B-cells are effectively depleted (Baumgartner et al., 1988, Storek et al., 2001) but circulating IgG levels drop slowly due to their long half-life in serum (Cohen and Freeman, 1960) and, survival of plasma cells even after myelodepletion (van Tol et al., 1996). In time, plasma cells would disappear resulting in a loss of protective antibodies against pathogens the patient had encountered previously (Wahren et al., 1984, Ljungman et al., 1990). Acute lymphoblastic leukaemic patients treated with high intensity regimes have also been associated with low level of immunoglobulins (Abrahamsson et al., 1995) and have prompted the initiation of re-vaccination post treatment in order to restore humoral immunity as soon as possible (Patel et al., 2007a, Patel et al., 2007b).

However, suboptimal response to immunisation has also been observed if intensive chemotherapeutic regimes had been administered (Feldman et al., 1988, Alanko et al., 1992, Borella et al., 1972, Hitzig et al., 1976b, Hitzig et al., 1976a, Katz et al., 1987, Layward et al., 1981, Ridgway et al., 1991, Weisman et al., 1987, Borella and Webster, 1971, Rautonen et al., 1986). In one study, low protective antibodies to measles and poliovirus were observed even one year after completion of therapy (Smith et al., 1995). Thus, antibody levels may be depressed long after cytotoxic therapy from the loss of pre-existing B-cells, slow B-cell regeneration and loss of supporting cells, for example CD4<sup>+</sup> T-cells, which are essential for B-cell activation.

#### 2.4.2 Innate immune system

The ‘ancient’ arm of the immune system, components of the innate immune system is normally the first to deal with an invading pathogen. Utilising both cellular and acellular components, pathogens are inactivated early to prevent further spread.

#### 2.4.2.1 Cellular compartments

Cells that make up this component include macrophages, monocytes, NK cells and the granulocytes (neutrophils, eosinophils and basophils). Early establishment that the depth and duration of neutropaenia is associated with serious bacterial infection (Schimpff et al., 1971, Bodey et al., 1966) has led to the use of absolute neutrophil counts as a measure of immunosuppression in this patient group (Freifeld, 1993, Pizzo, 1993, Chanock and Gorlin, 1996). Of course the quantity and quality of the other phagocytic cells (Losito et al., 1978, Hersh et al., 1966) are also invariably affected by the cytotoxic treatment but too few studies have been published that therapy induced neutropaenia remain the most important factor in infection risk (Freifeld, 1993, Pizzo, 1993).

In addition to depletion in numbers, neutrophil dysfunction after exposure to cytotoxic drugs has been reported in *in vitro* studies. Neutrophils isolated from healthy volunteers showed impaired superoxide generation, microbiocidal activity and phagocytosis (Vaudaux et al., 1984, Whittaker et al., 1975, MacFadden et al., 1985, Pruzanski et al., 1983, Pickering et al., 1978, Hara et al., 1990). *Ex vivo* studies with neutrophils isolated from lung cancer patients have also demonstrated reduced superoxide generation activity and reduced Fc receptor expression upon treatment with antineoplastic drugs (Ichinose et al., 1986). It is however, important to acknowledge that in some instances, the underlying haematological malignancy has already contributed to defects in phagocytic cells. Leucocytes from myeloid leukaemic patients display abnormal adhesiveness, migration, phagocytic function and killing activity of *S. aureus* and *C. albicans* (Lehrnbecher et al., 1997, Tornay, 1967, Wilkinson et al., 1975, Goldman and Th'ng, 1973). Further, it is pertinent at this point to remember that the innate and adaptive immune cells are interdependent on each other for normal function. Abrogation or dysfunction of T- and B- cells could have profound effects on the cells of the innate immune system as well.

#### 2.4.2.2 Acellular compartments

The acellular compartments of the innate system circulate continuously and act as sentinels to detect the presence of microbes. Generally thought of as unspecific, these molecules, mostly plasma proteins, are designed to recognise parts of microorganisms, bind to them and send off signals to recruit cells to either destroy the microbe or phagocytose them. Prototypical molecules include members of the complement system

(classical-, alternate- and lectin- pathways), lysozyme, lactoferrin, fibronectin and the antimicrobial peptides.

In an immunocompetent individual, their immediate early response serve to curtail infection and send out signals to recruit cellular components of the immune system and initiate a cascade of activity leading to the resolution of current infection and ‘memory’ for recurrent encounters. In the absence of cellular components in the immunosuppressed patient, these plasma proteins potentially play a more important role in pathogen defence.

Mannose-binding lectin (MBL) deficiency has indeed been associated with infections in an immunosuppressed patient group (Molle et al., 2006, Mullighan et al., 2002, Neth et al., 2001, Peterslund et al., 2001) and have been suggested for replacement therapy (Valdimarsson, 2003, Valdimarsson et al., 2004, Brouwer et al., 2009, Petersen et al., 2006) although conflicting results remain (Frakking et al., 2011, Bergmann et al., 2003, Kilpatrick et al., 2003). Another classical acute phase protein, C-reactive protein (CRP) has been earlier suggested to be elevated in bacteraemia-related fevers in immunosuppressed patients (Hambach et al., 2002, Pihusch et al., 2006) however, other studies have found no correlation. In neutropaenic patients, procalcitonin (PCT) has also been demonstrated to discriminate bacteraemic-fevers from fevers of unknown origin (FUO) (Engel et al., 1999, Hambach et al., 2002, Pihusch et al., 2006, Schuttrumpf et al., 2003, Ruokonen et al., 1999) but several studies have highlighted the dynamic nature of PCT secretion (Engel et al., 1999, Jimeno et al., 2004, Fleischhack et al., 2000, Schuttrumpf et al., 2003).

Dynamics of stimulation and secretion seems to be the crucial factor in the efficacy of these proteins as biomarkers for infection-related fevers. This is hardly surprising since, the role of acute phase proteins are primarily pro-inflammatory, and transience, is the key to limit bystander cell/tissue damage at the expense of halting an infection.

### 3 AIMS OF THE THESIS

The overarching aim of this thesis was to extend our knowledge of the aetiology of, and susceptibility to infection in the neutropaenic patient. Here, we focus on an adult cohort rendered immunosuppressed after chemotherapy treatment for their underlying haematological malignancy.

To achieve this aim, the thesis was designed in three parts :

1. Detection and characterisation of viral infections via :
  - a. Evaluation of sampling methodology for nasal secretions (**Paper III**) and;
  - b. Development of molecular detection method to determine the prevalence of a select panel of viruses (**Paper II**).
2. Determine the contribution of microbial translocation towards sterile febrile neutropaenic episodes classified as ‘Fever of unknown origin (FUO)’ (**Paper IV**).
3. Investigate the role of innate host response for potential replacement therapy during neutropaenia (**Paper I**).



## **4 PATIENTS, MATERIALS AND METHODS**

### **4.1 PATIENTS AND INCLUSION CRITERIA**

The sub-studies/papers included in this thesis use material obtained from a single patient cohort but numbers in each paper vary depending on the material type and also on assay requirements. During a 26 month period (January 2008 to February 2010), adult patients with haematological disorders at the Karolinska University Hospital, Stockholm were, after informed consent, included in a cross sectional study where the inclusion criterion was chemotherapy-induced neutropaenia (absolute neutrophil count  $\leq 500/\text{mm}^3$ ). Patients that developed fever (auricular temperature  $>38.0^\circ\text{C}$  twice within an hour or  $\geq 38.5^\circ\text{C}$  at one occasion) were sampled within 72 hours from fever onset, whereas patients without fever were sampled upon routine medical appointments during the neutropaenia episode. The study was approved by The Regional Ethical Review Board in Stockholm, permit numbers 2007/1213-31/4 and 2008/1300-32.

In total, 144 febrile neutropaenic episodes from 124 patients and 47 afebrile neutropaenic episodes from 39 patients were included in the whole study.

### **4.2 MATERIALS**

#### **Paper I, II and IV**

In addition to samples collected for routine clinical investigations, two vials of whole blood in EDTA-tubes and one vial of nasal pharyngeal aspirates (NPA) in 2-3mL of saline were collected within 72 hours from the onset of neutropaenic fever or at a routine scheduled visit for neutropaenic patients without fever. A 150 $\mu\text{L}$  aliquot of whole blood was set aside at room temperature for flow cytometry analysis within 6 hours while plasma was prepared from the remainders by centrifugation and all study samples were stored at  $-80^\circ\text{C}$  until use.

Medical records were retrospectively acquired for all included patients. Data extracted included the characteristics pertaining to the patients (age, sex, fever duration and absolute neutrophil counts), underlying haematological malignancy, treatment regime,

routine clinical microbiological investigation results (where available) and C-reactive protein (CRP) concentrations at time of sampling.

### **Paper III – Flocked nasal swab**

98 paired samples of nasal and nasaopharyngeal secretions were obtained with the flocked nasal swab and NPA apparatus respectively at the same sampling occasion.

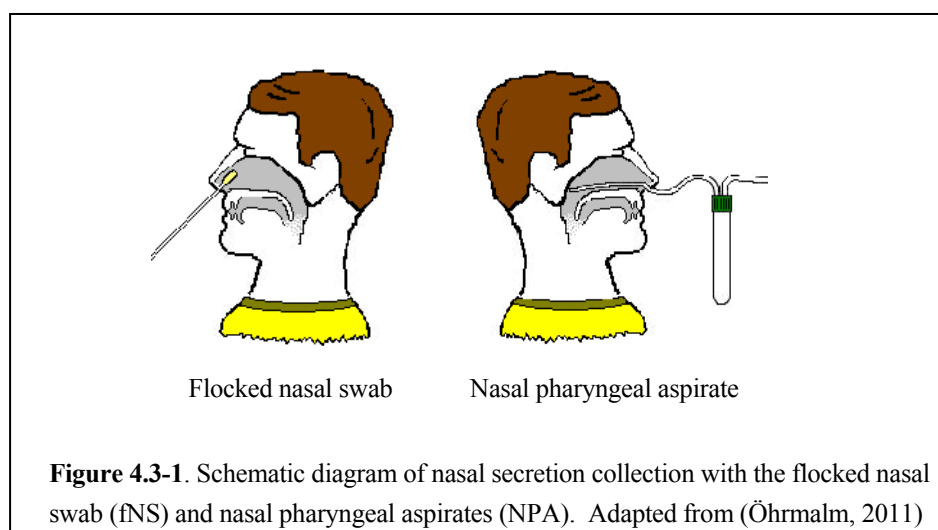
## **4.3 METHODS**

### **4.3.1 Flocked nasal swab and nasal pharyngeal aspirate collection**

#### **Paper III**

To minimise carryover from subsequent sampling, nasal secretion samples was first obtained with the flocked nasal swab (Copan cp552c) followed by nasal pharyngeal aspirate collection with a sterile catheter (Medioplast, no. 8).

Briefly, the fNS was inserted at least 2cm into each of the nostrils and rotated to collect as much secretions as possible thereafter, NPA samples were collected by inserting a sterile catheter into the posterior nasopharynx and, with gentle suction, secretions were collected and flushed with 2-3ml of physiological sodium chloride (Fig. 4.3-1). Both sample types were stored without additional media and transported to the laboratory within six hours. A sample suspension was prepared by shaking the fibre tip of the fNS in 500µl RPMI 1640 media, for 30 mins. Both sample types were stored in -80°C till use.



Viral nucleic acids were extracted from 400µl of NPA or fNS suspension sample with the BioRobot M48 using the MagAttract virus mini M48 kit (Qiagen GmbH, Germany) and eluted in 100µl of nuclease-free water.

#### **4.3.2 Quantitative PCR (qPCR) for detection of viruses**

##### **Paper I-II and IV**

Although described extensively only in papers II and III, data from the qPCR runs for virus detection was used extensively in papers I and IV as well. To avoid reinventing the wheel, published primers and probes for desired viruses were compiled and checked against curated sequences uploaded onto Genbank, NCBI (September 2008) with the selection criteria of pan-species coverage whilst retaining specificity. Where unavailable, in-house primers and probes were designed after sequence alignment and identification of conserved regions unique to the species.

All completed genomes of the target viruses were obtained from Genbank, NCBI (GenBank) and aligned using ClustalW and conserved regions were eyeballed. Additional sequences of the selected conserved region were added to the alignment for a construction of a consensus sequence. Primers and probes targeting the conserved region were designed with Primer express (Applied Biosystems) based on the consensus sequence. Table 4.3-1 lists the virus panel utilised as well as the origin of the PCR protocol, where it was not designed in-house.

Blood samples were screened for DNA viruses that might reactivate during immunosuppression for example, cytomegalovirus (CMV), Epstein-Barr virus (EBV), adenovirus (AdV) and other viruses reportedly detected in immunosuppressed patients, parvovirus B19 (B19) and BK polyomavirus. With the NPA samples, respiratory viruses circulating in the community were selected for the screening panel. These included human rhinovirus (HRV), human enterovirus (HEV), influenza virus A and B (FluA and FluB), respiratory syncytial virus (RSV), parainfluenza virus 1-4 (PIV 1-4), human coronavirus (HCoV) and human metapneumovirus (HMPV).

**Table 4.3-1.** Virus screening panel used in this study

Virus	Gene target	Dye chemistry	Origin
<b>Blood samples</b>			
Cytomegalovirus	Polymerase	Vic-MGB	Yun et al. (Yun et al., 2003)
Epstein-Barr virus	BNRF1	Ned-MGB	Niesters et al. (Niesters et al., 2000)
Adenovirus	Hexon	6FAM-MGB	Gustafsson et al. (Gustafson et al., 2008)
BK polyomavirus	VP2,3	Cy5-BHQ3	This study
Parvovirus B19	NS1	6FAM-MGB	This study
<b>Respiratory samples</b>			
Human rhinovirus	5' UTR	6FAM-BHQ1	Lu et al. (Lu et al., 2008)
Human enterovirus	5' UTR	Cy5-BHQ3	Nijhuis et al. (Nijhuis et al., 2002)
Influenzavirus A	Matrix	Vic-TAMRA	WHO/CDC
Influenzavirus B	HA	6FAM-MGB	Tiveljung-Lindell et al. (Tiveljung-Lindell et al., 2009)
Respiratory syncytial virus	Matrix	Cy5-BHQ3	Brittain-Long et al. (Brittain-Long et al., 2008)
Parainfluenzavirus 1	HN	Ned-MGB	Terlizzi et al. (Terlizzi et al., 2009)
Parainfluenzavirus 2 and 3	HN	Ned-MGB	This study
Parainfluenzavirus 4	Phosphoprotein	Ned-MGB	This study
Human coronavirus	Polyprotein 1ab	Vic-MGB	This study
Human metapneumovirus	Nucleoprotein	Ned-MGB	This study

Taqman® real time qPCR assays were used to screen for all the viruses. Quantitation of DNA viruses was achieved with the construction of a plasmid DNA controls containing target sequences for respective viruses whereas qualitative analyses were performed for the respiratory viruses.

### **4.3.3 Fluorescence-activated cell sorting**

#### **Paper I, II and IV**

In addition to granulocyte counts performed in the clinical laboratories, T-cells, B-cells and NK cells were counted as a measure of immune-suppression. The BD multitest™-IMK kit with BD Trucount™ tubes (BD Biosciences) were used to measure CD3+/CD4+ T helper cells, CD3+/CD8+ T cytotoxic cells, CD3-/CD19+ B cells, CD3-/CD16+/CD56+ NK cells. Briefly, 50µL of fresh whole blood is mixed with the BD multitest reagents and incubated for 15minutes before lysis and analysis.

### **4.3.4 ELISA**

#### **Paper I and IV**

Commercially available ELISA kits were utilised for measurements of Human plasma MBL oligomer (Bioporto, Denmark) and Human plasma sCD14 (R&D systems Inc. Minneapolis, USA) concentrations.

### **4.3.5 Single Nucleotide Polymorphism (SNP) identification**

#### **Paper I**

In paper I, SNP genotyping was performed to evaluate the contribution of polymorphisms within *MBL2* to the frequency or type of infection. Six SNPs previously reported to impact secreted MBL protein concentrations (Madsen et al., 1995) were selected and a nested PCR approach was undertaken to identify the alleles in these loci. Cytopenia in this patient cohort limits the amount of DNA available for typing hence, a nested PCR approach coupled with capillary sequencing allowed efficient use of the restricted starting material.

Multiple sequence alignments against the reference *MBL2* were subsequently performed to locate each SNP locus and the allele eyeballed and recorded by hand.

### **4.3.6 Chromogenic Limulus Amoebocyte Lysate (cLAL) assay**

#### **Paper IV**

Measurement of bacterial lipopolysaccharides (LPS) was performed with a commercially available (Lonza group Ltd., Switzerland) chromogenic Limulus

amoebocytes lysate assay (cLAL) but modified with diazo coupling of the chromogenic substrate (Novitsky, 1998) before absorbance measurements.

Using pyrogen-free reagents and consumables (Lonza), heat inactivated plasma were aliquoted into a flat-bottom 96 well plate and an initial background absorption measurement was taken at this point to correct for variation in the patient's plasma. With the exception of diazo-coupling in the final steps, the assay was subsequently performed as recommended by the manufacturer. All absorption measurements are thus taken at 540nm, the maximal absorption for the azo dye. LPS quantitation in the plasma samples were determined against a standard curve of endotoxin standards provided in the kit.

The commercial assay as it was designed was unsatisfactory due to a lack of consideration of background absorbance from components within human plasma. Further, the absorption wavelength of the chromogenic substrate (p-nitroaniline) was not well separated from that of normal human plasma potentiating unreliable quantitation of LPS within the sample. Derivatizing p-aniline (pNA) into its diazo form results in a brilliant magenta-coloured dye, measured at 540nm, thus limiting interference from plasma components. Subtraction of background absorption at the start of the assay also helps to correct for absorption intrinsic in the plasma itself.

#### **4.3.7 Statistical analysis**

##### **Paper I-IV**

With the exception of regression analyses, all statistical analyses were made with the Prism suite (Graphpad Inc., CA, USA). Fisher's exact test was used for investigating categorical data in the patient's general characteristics. Non-categorical data were analysed either with Mann-Whitney or Kruskal-Wallis test, depending on the number of groups. Correlations were calculated using Spearman's rank test or Pearson's correlation after log transformation. In paper I, Wilcoxon signed-rank test was used for paired samples and Cohen's kappa was calculated as a measure of agreement of results obtained from the two sampling methods. All tests were two sided, with p-value < 0.05 considered statistically significant.

In paper II, forward conditional binary logistic regression analyses were performed for multivariate analysis of factors associated with fever as the dependent variable. PASW Statistics 18 was used for this purpose.

## 5 RESULTS AND DISCUSSION

The general characteristics of the whole cohort are described in table 5.1-1 and details for the patients included in the individual studies are described in the attached publications and manuscripts. Across all studies, no patient presented with clinically diagnosed invasive fungal infections.

**Table 5.1-1.** General characteristics of the entire cohort

Characteristics	Fever (n=144)	Without fever (n=47)	p-value
No. of females (%)	61 (42)	19 (40)	n.s.
Age [years], median (range)	58 (20-86)	62 (28-85)	n.s.
Underlying disease (%)			
Acute leukemia/MDS	68 (47)	23 (49)	n.s.
Chronic myeloid leukemia	0 (0)	1 (2)	n.s.
Chronic lymphocytic leukemia	6 (4)	4 (9)	n.s.
Non-Hodgkin lymphoma	45 (31)	14 (30)	n.s.
Myeloma	16 (11)	5 (11)	n.s.
Hodgkin's disease	6 (4)	0 (0)	n.s.
Others	3 (2)	0 (0)	n.s.
Cell count <sup>a</sup> [/mm <sup>3</sup> ], median			
Neutrophils	<100 (<100-500)	200	***
Lymphocytes	107 (2-1002)	437	***
Monocytes	43 (0.02-1337)	78	n.s.

**NOTE.** Characteristics are presented in number and percentage of each group. Continuous data are presented in medians, followed by range. Continuous data are dichotomized above and below the median. OR, odds ratio; CI, confidence interval; MDS, myelodysplastic syndrome.

<sup>a</sup> Except for the neutrophil count, data on cell count were only available from 62 of the fever episodes and 37 of the controls.

\*\*\* p<0.001 and n.s., not significant



## 5.1 VIRUS DETECTION AND CHARACTERISATION

### (Papers II and III)

Viral contribution to infection and febrile episodes in this patient group has not been well characterised previously and is rather relevant in view of the prolific use of broad-spectrum antibiotics forestalling potential bacterial infections. In their immunosuppressed condition, bacterial infections account for substantial morbidity and mortality (Meckler and Lindemulder, 2009, Bodey et al., 1966, Ellis, 2008) and while antibiotic administration is essential there remains a need for judicious use to limit the potential spread of antibiotic-resistant bacterial strains. Determining the aetiology of fevers in this patient group could allow the clinician to make informed decisions on the length or discontinuation of broad-spectrum antibiotics. To this end, we designed the study in two parts. First, we attempted to improve sampling of nasal secretions thereafter we assembled a panel of PCR assays targeting blood-borne and respiratory viruses.

Nasopharyngeal aspirates (NPA) had been the method of choice when sampling nasal secretions but this is wrought with operator-dependent variations and in paediatric cohorts, had been a reason for study rejection. Ease of collection, reduced equipment requirement, cost efficacy and ease of transport are some reasons favouring the use of the flocked nasal swab (fNS) instead of the nasopharyngeal aspirates. In **paper III**, a simple study was performed to ascertain the sample collection efficacy with 98 paired samples of fNS and NPA. Quantitative PCR (qPCR) for a panel of respiratory viruses and the human beta-actin gene was used to evaluate efficiency for virus sampling and general sampling efficacy respectively.

Collection method notwithstanding, in 20 of the 196 samples respiratory viruses were detected (table 5.1-2). Of these, 11 were common to both sampling methods, seven only with the NPA and two only with the fNS resulting in a sensitivity of 90% and 65% respectively. With the exception of only one sample, CT values for samples collected with NPA was lower than that of fNS suggesting that the NPA method was a better method for respiratory virus sampling. This trend was also reflected in the CT values for human beta-actin gene.

**Table 5.1-2.** Virus detection with the NPA and fNS sampling methods.

Virus	No. of samples in which virus was detected by:			Total no.	Mean difference* in Ct values (range)
	Both	NPA	fNS		
	methods	only	only		
Rhinovirus	7	2	0	9	3.2 (-2.0-6.5)
RSV A	1	1	1	3	5.8
RSV B	2	0	0	2	2.2 (5.6-9.9)
Influenza A	0	1	0	1	N/A
Influenza B	1	1	0	2	15.5
Bocavirus	0	1	0	1	N/A
Enterovirus	0	1	0	1	N/A
Metapneumovirus	0	0	1	1	N/A
Any virus	11	7	2	20	5.1 (-2.0-15.5)

\*The Ct value for the flocked nasal swab minus the corresponding Ct value for NPA.

The most glaring discrepancy in our study is of course, the depth and location from which we collect the NPA and fNS sample; with the NPA, the posterior nasopharynx and with the fNS, the outer nostrils. Attempts to reach the nasopharynx with the fNS would result in considerable discomfort to the patient and defeats the primary purpose of the study. Additionally, the suction force used with the NPA collection method probably provided better ‘scrubbing’ than simple ‘brushing’ with the fNS. Admittedly, the primary location for viral replication in most cases would not be within the nostrils and the initial optimism that respiratory viruses would be carried forward in the mucus was probably overestimated knowing that disrupted mucosal response is common in this patient group. Further, most other studies evaluating the efficacy of the flocked nasal swabs were performed in otherwise healthy patients that were symptomatic with respiratory tract infections. Even then, no clear association between sampling depth and viral detection could be observed (Abu-Diab et al., 2008, Heikkinen et al., 2002, Macfarlane et al., 2005, Stensballe et al., 2002, Sung et al., 2008). Studies that compared the use of nasal pharyngeal swabs and nasal pharyngeal aspirates, both of which sample the same region, also reported conflicting results (Ahluwalia et al., 1987, Cruz et al., 1987, Frayha et al., 1989).

The improved collection efficiency with the NPA, as evidenced by higher yields of epithelial cells, indicates the effects of another fundamental difference between the two methods. With the NPA, nasal secretions are collected directly into the collection tube

and remnants in the catheter are flushed with saline. In contrast, nasal secretions collected with the fNS need to be transferred upon arrival to the laboratory and we cannot exclude the possibility that despite all efforts, not all cells are dislodged from the swab into the swab suspension that we use for subsequent analyses. Overall, we concluded that the NPA method for sampling of nasal secretions should not be replaced in our adult iatrogenic-immunesuppressed patient group.

With the sampling method in place, we went on further to investigate the prevalence of viruses in the iatrogenic febrile neutropaenic adult in **paper II**. qPCR assays for a panel respiratory and blood-borne viruses were established and viral loads were determined with qPCR for CMV, EBV, AdV, parvovirus B19 and BK polyomavirus. Qualitative data was obtained with PCR for the respiratory viruses.

In our cohort, we saw that, of the 158 blood samples, 35 (22%) samples were positive for at least one blood-borne virus (table 5.1-3) but were all of low viral loads (<1000 copies/ml) with the exception of 6 samples that included one CMV finding at 1800 copies/ml, four BKV findings at 3500, 4800, 7000 and 38,000copies/ml and one B19 finding at 9500copies/ml. The relevance of these findings however, is debatable as these levels are lower than those expected with symptomatic disease.

**Table 5.1-3.** Presence of viruses in peripheral blood and nasopharynx in the 159 patients with neutropaenia.

Specimen	Virus	No. of positive (%)
Blood n=158	BK polyomavirus (BKV)	18 (11.4)
	Cytomegalovirus (CMV)	8 (5.1)
	Parvovirus B19 (B19)	6 (3.8)
	Epstein-Barr virus (EBV)	6 (3.8)
	Adenovirus (AdV)	1 (0.6)
No. of patients with at least one virus detected in blood		35 (22.2) <sup>a</sup>
NPA n=144	Rhinovirus (HRV)	11 (7.6)
	Adenovirus (AdV)	6 (4.2)
	Respiratory syncytial virus (RSV)	5 (3.5)
	Influenza A virus (IFA)	1 (0.7)
	Influenza B virus (IFB)	2 (1.4)
	Metapneumovirus (HMPV)	2 (1.4)
No. of patients with at least one virus detected in NPA		26 (18.1) <sup>b</sup>
No. of patients with at least one virus detected in any specimen		56 (35.2)

<sup>a</sup> Percentage of total sampling occasions where blood was investigated

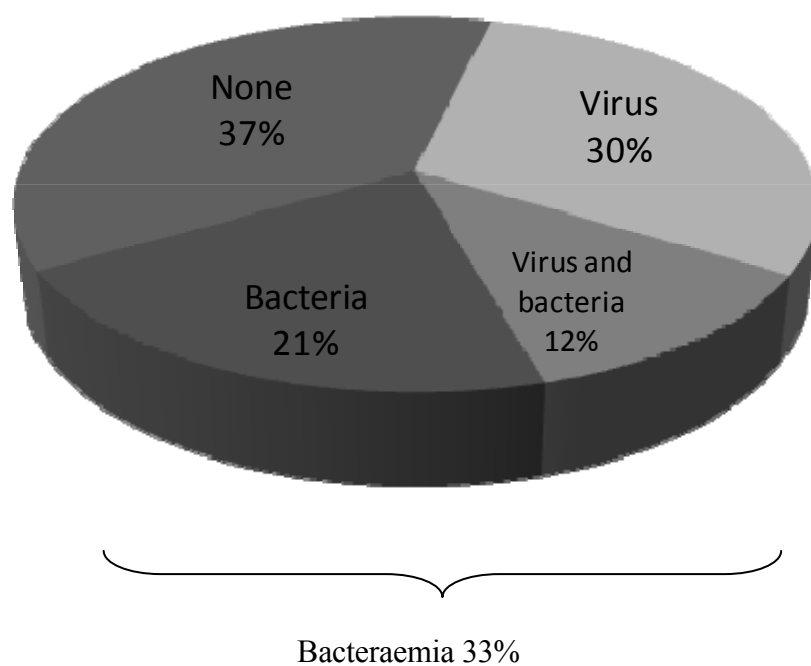
<sup>b</sup> Percentage of total sampling occasions where NPA was investigated

Correlation of viral loads with disease is problematic, especially with DNA viruses capable of latency, as is the case with the CMV and EBV. This is further confounded by reports that EBV DNA has been observed in healthy individuals (Lay et al., 2010). Peripheral blood is widely accepted as a sterile environment and any pathogen detected should be an indication of an ongoing or ensuing infection which might or might not manifest as symptomatic disease. At the point of detection, the immunocompetent individual could already have mounted an effective anti-viral response hence, observable disease does not occur. However, immunosuppression places the patient at double-risk; inability to stem the start of an infection and, as a result, inability to present with clinical symptoms of the disease. A contentious suggestion, no doubt, but further evaluation is necessary before dismissal.

Respiratory viruses were detected in 26 of the 144 NPA samples screened (table 5.1-3) and the prevalence of the respective viral species corresponded well with a study on community acquired pneumonia in the same geographical area (Johansson et al., 2010). Our findings also agree with a previous study suggesting that temporal occurrence of viral infections in immunocompromised patients tend to mirror that of the general community (Englund et al., 1999). It was an unfortunate oversight not to have included a control population of matched-healthy adults during the sampling period of the study, as a proxy of the circulating respiratory viruses in the particular time and space. Unlike the blood, the respiratory tract is constantly exposed to environmental contaminants and association between the detection of viral particles and disease is inherently more difficult. Yet, the likelihood that we would be able to detect transient contaminations is presumably rather low too. Either way, the study would benefit from information depth available with quantitation of viral loads and sequencing data to follow time-variable changes in viral species/strain findings.

When the patients were stratified based on febrile status (febrile or afebrile), significantly more viruses were detected in patients with febrile neutropaenia (42%) than afebrile patients (11%). Although tempting to immediately conclude that virus infections are associated with febrile episodes, we have to be mindful that despite efforts to match the afebrile patients, they remain less immunosuppressed as indicated by their leukocyte cell counts. With fever as the dependent variable, multivariate regression analysis to include cell counts and other confounding factors (with  $p\text{-value} < 0.10$ ) listed in table 2 was performed and, the association between fever and presence of virus remained. Moving ahead with caution, we conclude that febrile episodes with viral findings should be considered as infections till otherwise indicated.

The association between bacterial findings and febrile episodes is well established and 33% of the febrile neutropaenic episodes in our study are attributed to bacteraemia. In our study, we add to the field with our observation suggesting that, a further 30% of these febrile episodes are associated with viral findings (Fig. 5.1-1). The aetiology of fever in the last third of the group remains unknown and are thus categorised as patients with fever of unknown origin (FUO). This study would have benefitted with follow-up study samples to verify the association between viral findings and fever and, should definitely be considered in the future.



**Figure 5.1-1.** Documented microbiological findings in 123 febrile neutropaenic patients.

## **5.2 MICROBIAL TRANSLOCATION CONTRIBUTES TO EPISODES WITH FEVER OF UNKNOWN ORIGIN**

### **(Paper IV)**

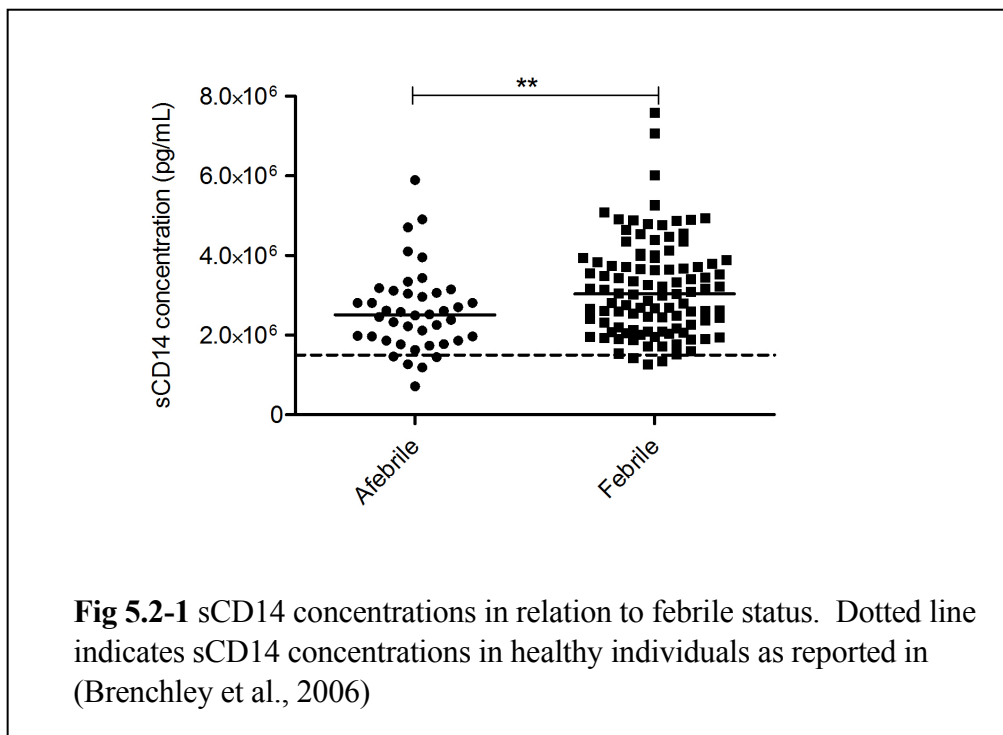
Despite extensive microbiological investigations, approximately 30% of febrile episodes in our cohort have no known cause, termed fever of unknown origin (FUO). Sterile fevers have of course been associated with the haematological malignancy itself or, as a result of the cytotoxic treatment these patients undergo. However, intestinal mucosal damage and the subsequent inflammation-mucositis is a common occurrence in our patient group as a result of cytotoxic agents administered for treatment of their underlying disease. Further, microbial translocation has been reported in studies with immunocompromised HIV-1 infected cohorts (Redd et al., 2009, Brenchley et al., 2006). This study then sets out to determine if fever in patients categorised as FUO, could be attributed to microbial translocation from the gastrointestinal tract.

Endotoxins more specifically, lipopolysaccharides (LPS), are commonly found on the outer membrane of Gram-negative bacteria and is used in this study as a measure of microbial translocation. Additionally, sCD14 was also measured concurrently to ascertain host response to LPS in this immunosuppressed patient cohort.

In our study, we observed elevated endotoxin concentrations, in concurrence with HIV-1 infected immunosuppressed cohorts (Brenchley et al., 2006) but not between the febrile and afebrile episodes. Further stratification of the febrile episodes based on microbiological findings revealed that despite the elevation across the cohort, episodes with documented bacteraemia (median=104.7 pg/ml [87.1pg/ml-112.3pg/ml]) had higher endotoxin concentrations compared with those of viral findings (median=97.3 pg/ml [85.45pg/ml-109.4pg/ml]). Endotoxin concentrations from FUO episodes (median=111.8 pg/ml [99.8pg/ml-134.9pg/ml]) were even higher than bacteraemia episodes, suggesting that there is similar, if not more, circulating bacterial products in this group.

Since fever is a manifestation of host response to LPS, the corresponding host sCD14 concentrations could explain the similarities in endotoxin concentrations between the febrile and afebrile episodes. As expected, sCD14 concentrations were significantly higher in the febrile episodes compared to the afebrile episodes suggesting that a host

response mediated through sCD14 binding of LPS has been initiated, leading to the development of fever as a symptom and implicating afebrile patients potentially as inadequate responders (Fig. 5.2-1). Stratification of the febrile episodes to focus on episodes with bacterial findings and the FUO episodes revealed similar sCD14 concentrations in both the FUO ( $3.20 \times 10^6$  pg/ml [ $2.29 \times 10^6$  pg/ml-  $3.80 \times 10^6$  pg/ml]) and Gram-negative bacteraemia ( $3.34 \times 10^6$  pg/ml [ $2.52 \times 10^6$  pg/ml-  $4.01 \times 10^6$  pg/ml]) episodes and that they were higher than those observed in episodes with Gram-positive bacteraemia ( $2.55 \times 10^6$  pg/ml [ $1.87 \times 10^6$  pg/ml-  $3.07 \times 10^6$  pg/ml]).



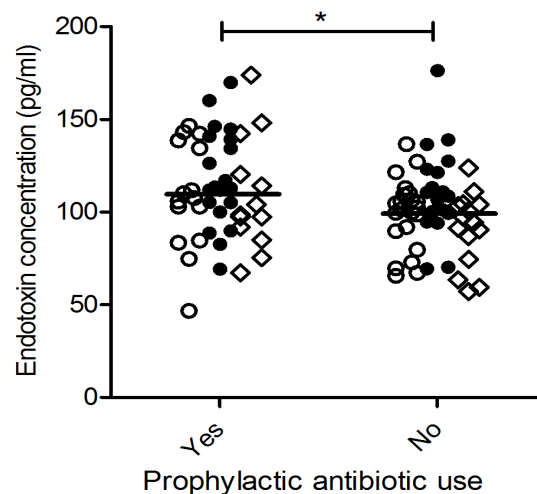
It is intriguing that the sCD14 concentrations do not mirror that of the endotoxin concentrations and could be attributed largely to the inherent immunosuppressed state of this patient group. Antineoplastic treatment these patients are subject to drastically reduces the monocytes and neutrophil populations and since these cells have been suggested to be the main sources of sCD14, a stunted response could be likely. The dual role of sCD14 further confounds explanation of the findings. In low concentrations, sCD14 transfers LPS to mCD14 and TLR4 to initiate a pro-inflammatory response however at high systemic concentrations, sCD14 acts as an attenuator to cell responses by shuttling LPS to lipoproteins (Kitchens and Thompson, 2005). sCD14 alternates between these two roles in a bid to launch a pro-inflammatory response in the presence of invading pathogens whilst attempting to limit bystander



tissue damage at the same time. To tease apart the role sCD14 is playing in our patients would require analyses of downstream proteins however, the transient and equilibrium-seeking nature of sCD14 might render this a rather difficult task.

Nonetheless, the elevated endotoxin and sCD14 concentrations observed in our patient group suggest that microbial translocation from the gastrointestinal tract has indeed occurred and could also contribute to the presentation of fever in the FUO episodes. The similarity of our findings to that of other immunosuppressed cohorts where microbial translocation has been established reinforces our conclusion.

Another interesting finding in this study was that the use of prophylactic antibiotics was associated with higher plasma endotoxin concentrations (Fig. 5.2-2). Liberal use of prophylactic antibiotics is commonplace in iatrogenic patients as a pre-emptive measure to bacterial infections yet our findings suggest that oral prophylactic antibiotics might predispose patients to increased risk of microbial translocation leading to fevers and subsequent delays in chemotherapy cycles. Without additional parameters such as type, dose and duration of prophylactic antibiotic use, it would be imprudent to speculate further.



**Fig 5.2-2** Endotoxin concentrations with (n=48) and without (n=55) prophylactic antibiotic use.(○) bacteria episodes; (●) FUO episodes; (◊) virus episodes

Earlier studies had presented conflicting results, reasons for which lie mostly with variations in methodology, sample sizes and inclusion criteria (table 5.2-1) and interpretation of our findings in context with earlier studies are inherently difficult.

**Table 5.2-1.** Studies on markers of microbial translocation in the chemotherapy-induced immunosuppressed patient.

Ref.	Numbers (Sampling regime)	Measures of microbial translocation	Microbial translocation?
(Harris et al., 1984)	100 (With and without chemotherapy)	Endotoxin with cLAL assay <sup>1</sup> with OD at 405nm as quantitation.	No
(Tancrede and Andremont, 1985)	680 (Faecal samples twice a week after chemotherapy and peripheral blood samples at onset of neutropaenia)	Concurrent positive bacterial cultures from blood and faeces.	Yes, observed in 55/64 documented Gram-negative bacteraemia episodes.
(McCartney et al., 1987)	10 (Daily, post chemotherapy)	Endotoxin with cLAL assay <sup>1</sup>	Unclear. Endotoxaemia was associated with Gram-negative bacteraemia but the reverse is not always true.
(Gunther et al., 1995)	94 (Fever onset, , day 1,2 and 6 thereafter)	Endotoxin with cLAL assay <sup>1</sup>	Unclear. Mean endotoxin concentration were similar with and without bacteraemia.
Wong, M (current)	93 (within 3days of febrile neutropaenia onset)	Endotoxin with cLAL assay <sup>1</sup> and sCD14.	Yes. Both endotoxin and sCD14 concentrations were higher in FUO episodes than in Gram-negative bacteraemia episodes.

<sup>1</sup> Chromogenic *Limulus* amoebocyte lysate assay.

<sup>2</sup> Optical density units

### 5.3 *MBL2* POLYMORPHISMS AND INFECTION SUSCEPTIBILITY

#### (Paper I)

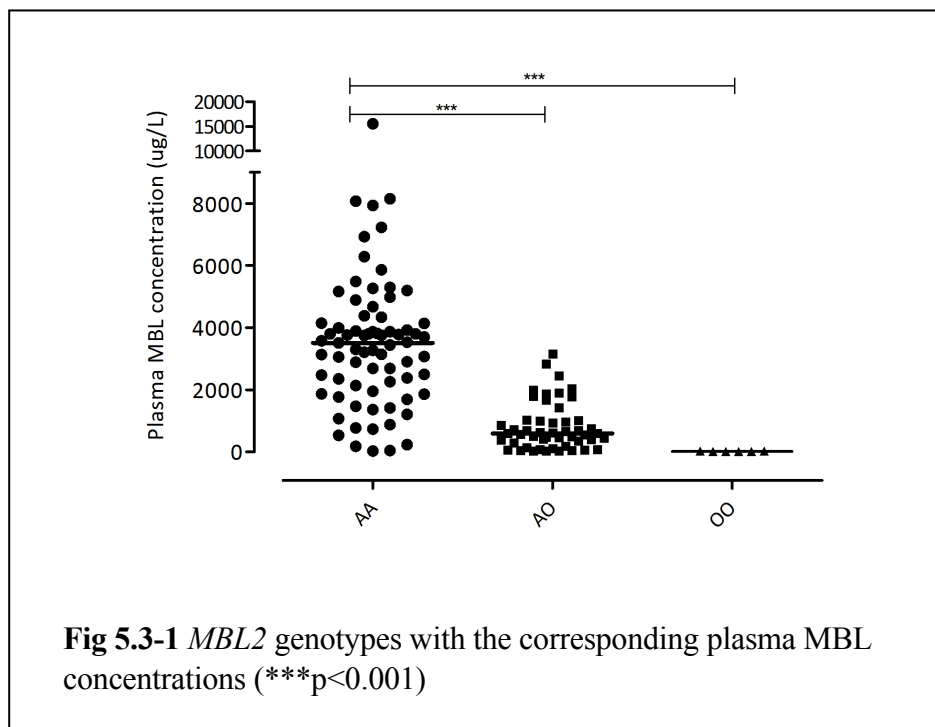
Infection episodes are common in this patient group during the neutropaenic phase and account for significant mortality (Pizzo, 1999). This is largely due to the immunosuppression from antineoplastic treatment the patients undergo for their underlying haematological disease. Promising results from previous studies had suggested that patients deficient in secreted mannose-binding lectin (MBL) could be more susceptible to infections (Molle et al., 2006, Mullighan et al., 2002, Neth et al., 2001, Peterslund et al., 2001). In fact, the use of recombinant MBL has been put through phase I clinical trials in Denmark (Petersen KA). The results from our study however show that in a Swedish adult iatrogenic patient cohort, neither *MBL2* SNPs nor plasma MBL levels did not correlate to infection risk or type.

In 96 febrile neutropaenic episodes and 33 afebrile neutropaenic episodes, plasma MBL concentrations were measured and *MBL2* SNP genotypes were determined. We first established that the allele frequency of the six *MBL2* SNPs and the corresponding haplotypes in our patient group were in Hardy-Weinberg equilibrium with a reference Danish population (Madsen et al., 1995) (table 5.3-1).

**Table 5.3-1.** *MBL2* genotype frequencies observed in this cohort (n=108).

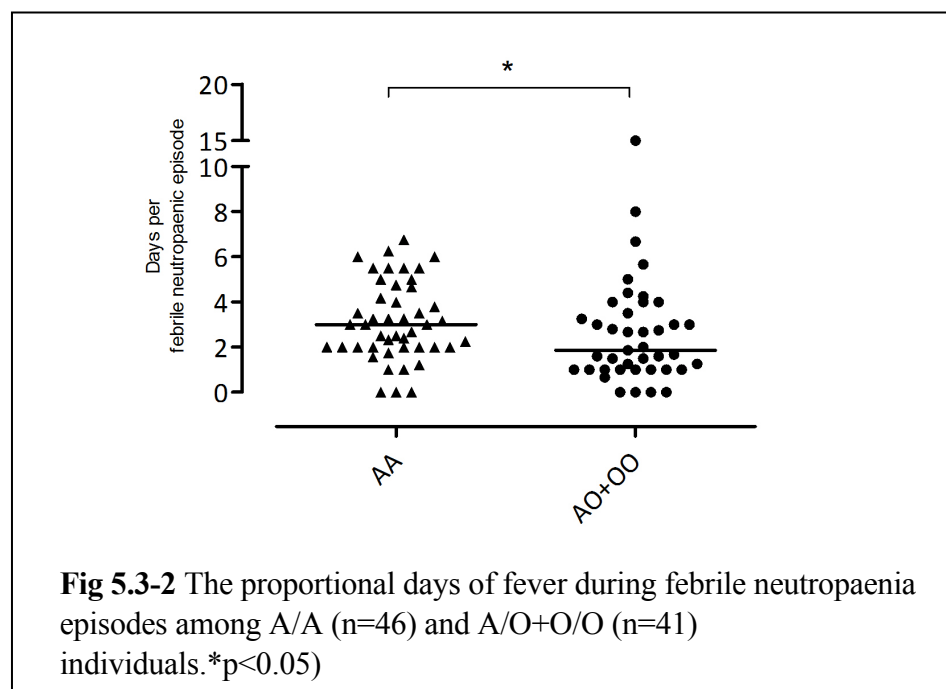
Promoter			Exon 1			Total	
dbSNP ID	allele	n (%)	dbSNP ID	allele	n (%)	Genotype	n (%)
rs11003125	L/L	47 (43.5)	rs5030737	A/A	96 (88.9)	A/A	63 (58.3)
	L/H	46 (42.6)		A/D	12 (11.1)	A/O	39 (36.1)
	H/H	15 (13.9)		D/D	0 (0.0)	O/O	6 (5.6)
rs7096206	Y/Y	67 (62.0)	rs1800450	A/A	75 (69.4)		
	Y/X	34 (31.5)		A/B	30 (27.8)		
	X/X	7 (6.5)		BB	3 (2.8)		
rs7095891	P/P	69 (63.9)	rs1800451	A/A	104 (96.3)		
	P/Q	31 (28.7)		A/C	4 (3.7)		
	Q/Q	8 (7.4)		C/C	0 (0.0)		

We further ascertained the correlation of the *MBL2* SNPs to plasma MBL concentrations and could be used to predict MBL deficiencies ( $p < 0.001$ ). Briefly, plasma MBL concentrations were drastically reduced in the presence of SNPs within the exon 1 coding region and the X/Y SNP in the promoter region playing only a secondary role (Fig 5.3-1). With this in mind, subsequent statistical analyses with clinical parameters were performed only with the coding region genotypes (A/A, A/O, O/O) to be statistically meaningful.



The two main clinical endpoints determined for this study were infection type and frequency, with presentation of fever as an indicator for infection. An episode-centric approach was taken for analyses with infection type among the febrile episodes without SNPs in the coding region (A/A). No difference in plasma MBL concentrations was observed between episodes with bacterial findings, bacterial or viral co-findings, and viral findings. In fact, plasma MBL concentrations were similar regardless of microbiological findings. This finding mirrors that of CRP concentrations, another acute phase protein, and could indicate disruptions in the acute phase response, presumably as a consequence of the antineoplastic treatment these patients have undergone. Interestingly, while MBL concentrations did not vary with the presentation of fever, CRP concentrations did, leading to postulations on the varied functions between these two proteins.

Using fever as an indicator of infection, we further performed patient-centric analyses to determine associations between *MBL2* coding region genotypes (A/A, A/O and O/O) and risk of fever and fever duration. No correlation was observed with *MBL2* genotypes and the risk of fever but a significant reduction in fever days per neutropaenic episode was observed in patients with the A/O and O/O genotype. The median number of febrile days per neutropaenic episode for A/A individuals was 3 days (IQR=2.0-4.7) compared to 1.8 days (IQR=1.0-3.8) for A/O and O/O individuals. Antibiotic use, which corresponds closely to fever duration, revealed the same trend with A/A and A/O+O/O individuals (Fig. 5.3- 2).



This finding adds to the body of studies with conflicting results regarding the relevance of MBL (Bergmann et al., 2003, Kilpatrick et al., 2003, Peterslund et al., 2001, Vekemans et al., 2007, Martinez-Lopez et al., 2009, Klostergaard et al., 2010, Mullighan et al., 2002) among immunosuppressed adults with underlying haematological disorders (Table 5.3-2).

**Table 5.3-2.** Studies on the relevance of *MBL2* and MBL protein concentrations in the chemotherapy-induced immunosuppressed adult patient.

Ref	Country, time period	Underlying disease (neutropaenia)	Numbers (Sampling regime)	Fever	Association to Infection	MBL measures
(Peterslund et al., 2001)	Denmark, N.A.	Mixed haematological malignancies (N.A. )	54 (Before chemotherapy)	N.A.	Yes	MBL protein
(Kilpatrick et al., 2003)	Glasgow UK, N.A.	Mixed haematological malignancies (1000cells/mL)	134 mixed bone marrow conditioning and chemotherapy. (Before treatment, neutropaenia onset, after neutrophil recovery)	No.	Yes. MBL<=0.1ug/ml had highest frequency of major infections.	MBL protein
(Bergmann et al., 2003)	Denmark, 1990-1993	Acute myeloid leukaemia (N.A.)	80 (Day of antineoplastic therapy initiation and weekly thereafter till day 28 )	No.	No.	MBL protein
(Molle et al., 2006)	Denmark, 1994-2003	Multiple myeloma (N.A.)	138 VAD <sup>1</sup> or Cydex <sup>2</sup> treated (N.A.)	N.A.	No	<i>MBL2</i> genotype
(Klostergaard et al., 2010)	Denmark, 1993-2004	Acute myeloid leukaemia (N.A.)	190 (Bone marrow aspirates)	N.A.	No.	<i>MBL2</i> genotype

(Mullighan et al., 2002)	Australia, 2002-2006	Mixed malignancies (Not available)	142 sibling HLA matched allo-HSCT (Pretransplantation conditioning, day 0,14,28 post transplantation)	No.	Yes. A/O and O/O are weak risk factors for infections	<i>MBL2</i> coding region genotype and MBL protein
(Vekemans et al., 2007)	Not available, 2001-2003	Mixed haematological malignancies (<500cells/mL)	255 (Day 1 of chemotherapy or 3 days before chemotherapy)	No.	Yes..	<i>MBL2</i> genotype and MBL protein
(Wong et al., 2012)	Sweden, 2008-2010	Mixed haematological malignancies (<500cells/mL)	82 (within 3days of febrile neutropaenia onset)	No but among febrile episodes, ratio of fever per neutropaenic day was higher in A/A than A/O+O/O patients.	No.	<i>MBL2</i> genotype and MBL protein

<sup>1</sup> Vincristine, doxorubicine, dexamethasone

<sup>2</sup> Cyclophosphamide and dexamethasone

Indeed, promising results from previous studies had been in paediatric oncology cohorts (Neth et al., 2001) but a recent meta-analysis of these studies has revealed that MBL deficiency could not be identified as risk factor for infection in these patients (Frakking et al., 2011). A similar meta-analysis for adults should be performed although our findings make it apparent that the relevance of MBL is limited and further clinical trials are unnecessary.

What's more fascinating is the general elevation of MBL concentrations in this patient group. Unfortunately, a healthy control group was not included in this study but simple comparisons with previous studies reporting MBL concentrations in blood bank donors suggest that MBL concentrations are elevated in our study. This finding was presented in some earlier studies (Bergmann et al., 2003, Kilpatrick et al., 2003) but was regrettably not further discussed. In vitro studies conducted with human colon adenocarcinoma cell lines demonstrated that MBL does indeed bind directly to transformed cell lines exhibiting aberrant glycosylation patterns (Muto et al., 1999). Conceivably, the preceding chemotherapy treatment the patients underwent could have already stimulated an increase in MBL concentrations as a means to facilitate removal of dead cells via phagocytosis. Thus, the MBL response to ensuing infection could have been masked. Put together, further studies regarding the function of MBL would be interesting but studies leading to therapeutic benefit of human recombinant MBL in this patient group is doubtful.



## 6 CONCLUSION AND FUTURE PERSPECTIVES

With these iatrogenic neutropaenic patients, we have attempted to further dissect the aetiology of the fever. In the neutropaenic patient, fever is the foremost and sometimes, only indication of infection with other signs of inflammation minimal or even absent. Treatment for their underlying hematological malignancy with cytotoxic drugs renders these patients highly susceptible to pathogenic infections which, would have been easily cleared in a healthy individual. The use of prophylactic broad-spectrum antibiotics has been imperial in preventing fatal bacterial infections but we would like to put forward a future case for better informed use of antibiotics after acute-empirical assessment via discrimination between infectious and non-infectious fevers and, bacterial and non-bacterial infections.

Bacteraemia in this patient group is well established (Klastersky et al., 2007, Whimbey et al., 1987, Viscoli et al., 2005) and we had no intention of disputing the fact however, we propose that viral contribution to infections or fever should be better studied. Aside from reports of reactivation of latent DNA viruses for example, CMV (Holmberg et al., 1999, Metselaar and Weimar, 1989, Rajcani, 2007), and EBV (Babcock et al., 1999, Baldanti et al., 2000, Crawford et al., 1981, Yao et al., 1985), the panorama of viral infections in the immunosuppressed patient that have not undergone HSCT, is not well studied. During immunosuppression, patients are not often confined to the hospital and are thus potentially exposed to viruses to the same extent as the general community.

**Our work in paper II describes the prevalence of nine common respiratory viruses and show that it is congruent with the general prevalence in this geographical population. Additionally, an association between virus detection and fever was observed suggesting viral contribution to 30% of neutropaenic febrile episodes.** Although significant, we cannot ascertain that the association was causative. qPCR is a highly sensitive method for viral detection but its strength is sometimes its bane. Ability to detect minute quantities of viral nucleic acid does not necessarily mean a viral infection has occurred. In fact, determining the viral copy numbers and its relation to viral infections is rather tricky. With blood samples, one assumes a sterile environment hence the presence of any viral nucleic acid warrants concern yet, it is suggested that a clinical threshold is necessary to constitute an infection (Bai et al., 1997, Baldanti et al., 2000, Jebbink et al., 2003, Kimura et al., 1999, Lay et al., 2010,

Limaye et al., 2001, Mullier et al., 2009, Rowe et al., 1997, Wagner et al., 2004) . In the respiratory tract, where one is constantly exposed to environmental factors, it is perhaps even less surprising that viral nucleic acids are detected. To further confound the issue, infection with some respiratory viruses like HRV is not always associated to symptomatic fever (Jansen et al., 2011, Wright et al., 2007). The key question really is how we can correlate viral nucleic acid detection to viral infection. Longitudinal studies with samples taken regularly could be a means where viral load can be tracked in relation to the patient's symptoms. Detection of viral replication intermediates could be another option to demonstrate active replication within the host (Bannister et al., 2010, de Paula et al., 2009, Vaughan et al., 2008, Zawilinska et al., 2006, Zawilinska et al., 2008). Regardless of the study design, the fact remains that in most individuals, there is a threshold of viral copy numbers only beyond which, viral infection takes place with the corresponding symptoms. Nonetheless, for the purposes of this thesis, viral findings are assumed to be viral infections and this categorisation is used in papers I and IV.

Despite 33% of the febrile episodes attributed to bacteraemia and a further 30% to viral findings, 37% of febrile episodes still have no attributable cause (Fig. 5.1-1). Drug and treatment related fever (DTRF) is of course a possibility and often suggested where fever of unknown origin (FUO) is documented. The observed microbial translocation in another immunosuppressed group of HIV-1 infected patients suggests that a similar situation could occur in our patients. **In paper IV, we show that microbial translocation does indeed occur in our cohort and that fever in FUO episodes can be attributed to microbial translocation.** The association of sCD14, but not endotoxins, to fever suggests that some level of immune response is required in order to launch a cascade, leading to fever as an observable symptom. Antineoplastic treatment these patients undergo, primarily affects the cellular components of the immune system rendering acellular components of the innate immune system, such as MBL, potentially important in the role of microbial defence. The ability to predict infection susceptibility from MBL genotype is appealing but in our hands (**paper III**), we saw **no association between *MBL2* genotypes to frequency or type of infection.**

Put together, we,

- a. Suggest that 30% of febrile neutropaenia could be associated to viral findings;
- b. Show that microbial translocation contribute to fever in FUO episodes;
- c. Show no association between *MBL2* genotypes and infection suggesting recombinant therapy is not relevant in this patient group.

The critiques to this study are naturally, many. Working with humans, limit the amount of experimental manipulations and highlights the need for good study design.

Although all patients comply with the criteria of immunosuppression, the heterogeneity of their underlying haematological disease is not ideal. By virtue of their differing disease, treatment varies and potentially alteration of their immune system as well.

Much is also to be desired regarding the selection of appropriate controls and stringency in matching them, especially with regard to immunosuppression. As a cross-sectional study, it has fulfilled its purpose of offering a snapshot and allowing exploration of possible correlates to fever. Moving forward, a longitudinal study with samples taken before chemotherapy and at various timepoints thereafter should be performed to address fever causation.

Nonetheless, at this juncture, we put forward the notion that when faced with a febrile neutropaenic patient, in addition to the usual suspects of malignancy, drug and infectious complications of bacteraemia and fungaemia, viral infections and microbial translocation should be added to the lineup. The need for diagnostic tools and biomarkers that can effectively distinguish between these causes is thus vital for the administration of appropriate treatment modalities.

## 7 POPULAR SCIENCE SUMMARY

There has been drastic improvement in the way we treat blood cancers but the very treatment we use is toxic both to the malignant cancer cells as well as to the cells in our immune system. Nonetheless, this is a necessary evil and the only recourse is to treat the ensuing infections as they occur, with antibiotics and supportive care.

Most of the infections faced by patients immunosuppressed from the cytotoxic chemotherapy are from bacteria we encounter daily. Normally, harmless, infections in the immunosuppressed patient can potentially be fatal hence, antibiotics are usually prescribed upon manifestation of fever or, even in anticipation of infection. This has led to much concern regarding the spread of antibiotic-resistant bacteria and the fear that we may soon run out of 'new' antibiotics capable of killing these antibiotic-resistant strains of bacteria looms near.

In an effort to limit the unnecessary use of antibiotics, we have set out to determine the prevalence of viral infections in this patient group. Should we be able to accurately diagnose and differentiate between the various infection types, we should hopefully then be able to administer the relevant drugs for bacterial, viral or fungal infections. In our study with immunosuppressed patients, we have found viruses in 30% of the fever episodes where neither bacteria nor fungi are detected. We thus conclude that these fever episodes might be attributed to viral infections. This 30% with virus findings together with another 33% with bacteria findings still leave us with a remaining 37% of fever episodes where no pathogen could be detected.

Another side effect of the cytotoxic chemotherapy these patients encounter is the disruption of the lining in their gastrointestinal tract. This lining accounts for a large part of our immune system and works daily to prevent the bacteria we harbour in our lower intestines from entering our bloodstream. Disruption of this layer would naturally put us at risk and indeed, we see that in the patients that have fever but no pathogen detected, higher levels of bacterial products are observed in their blood. Together with the virus findings, we suggest possible causes for the fever episodes experienced by these patients.

Lastly, we also investigated the specific role of a protein in the immune system, mannose-binding lectin (MBL) against infection and fever in our patients. The availability commercially makes this protein an attractive choice as we could potentially administer it to the patients, helping them defend against infections while reducing antibiotics use. Unfortunately, in our study, we did not see any protective MBL effect in our patients.

In conclusion, we have added to the panorama of infectious agents and bacterial products implicated during fever episodes in the chemotherapy-induced immunosuppressed adult patient and have further discouraged suggestions for MBL replacement therapy.

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