A study of the kinetics of cytomegalovirus (CMV) DNA and Immediate early (IE) -mRNA levels in patients with CMV reactivation following bone marrow transplantation

Dissertation submitted as part of fulfilment for the M.D. (Branch-IV Microbiology) Degree examination of the Tamil Nadu Dr.M.G.R.Medical University, to be held in April 2014

CERTIFICATE

This is to certify the dissertation entitled, "A study of the kinetics of cytomegalovirus (CMV) DNA and Immediate Early (IE)-mRNA levels in patients with CMV reactivation following bone marrow transplantation" is the bonafide work of Dr. Sangeeta Susan Thomas toward the M.D (Branch –IV Microbiology) Degree examination of the Tamil Nadu Dr.M.G.R Medical University, to be conducted in April 2014.

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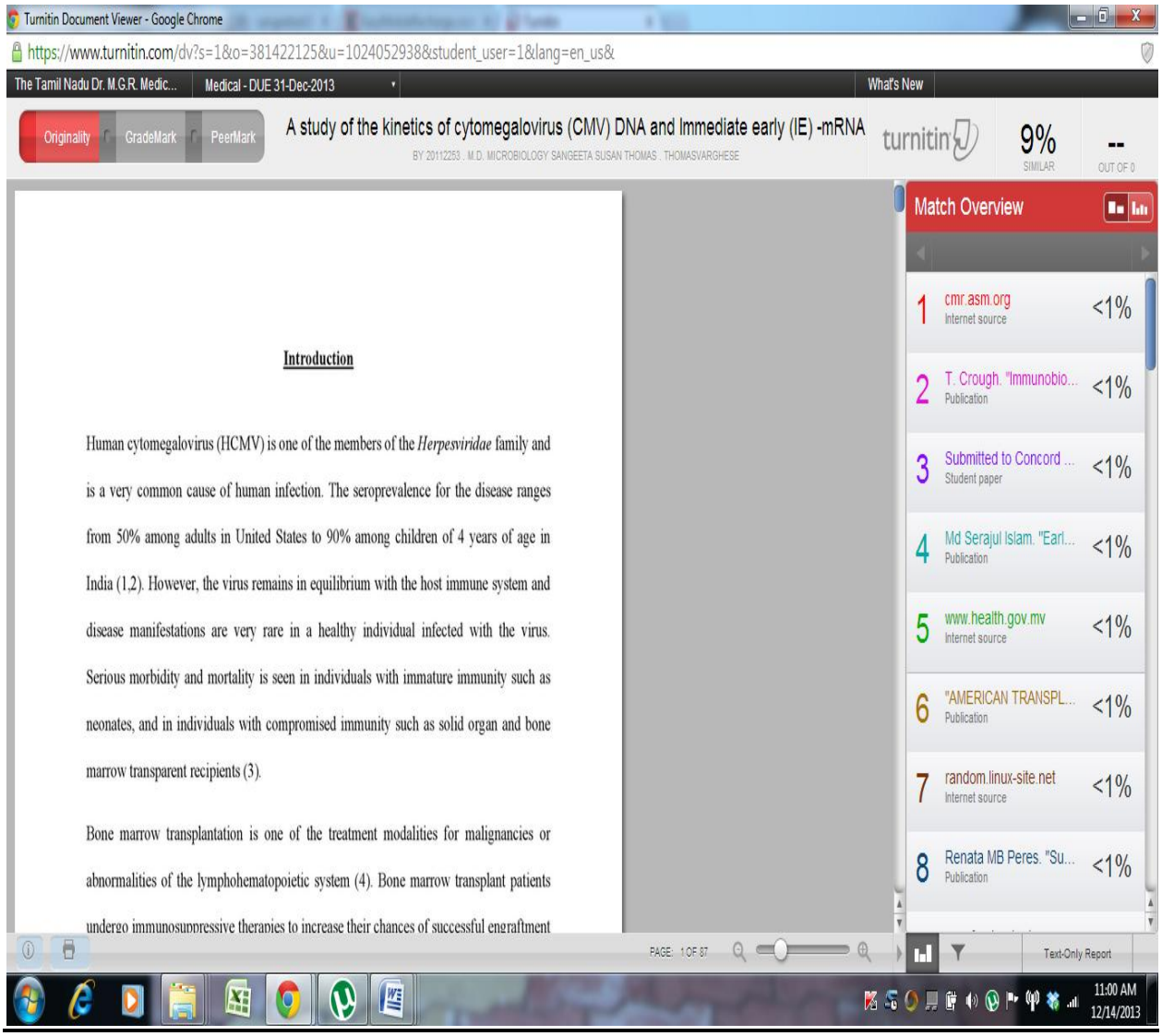
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Medical - DUE 31-Dec-2013
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A study of the kinetics of cytomegalovirus (CMV) DNA and Immediate early (IE) -mRNA
BY 20112253 . M.D. MICROBIOLOGY SANGEETA SUSAN THOMAS . THOMASVARGHESE
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Introduction
Human cytomegalovirus (HCMV) is one of the members of the Herpesviridae family and is a very common cause of human infection. The seroprevalence for the disease ranges from 50% among adults in United States to 90% among children of 4 years of age in India (1,2). However, the virus remains in equilibrium with the host immune system and disease manifestations are very rare in a healthy individual infected with the virus. Serious morbidity and mortality is seen in individuals with immature immunity such as neonates, and in individuals with compromised immunity such as solid organ and bone marrow transparent recipients (3).
Bone marrow transplantation is one of the treatment modalities for malignancies or abnormalities of the lymphohematopoietic system (4). Bone marrow transplant patients undergo immunosuppressive therapies to increase their chances of successful engraftment
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ACKNOWLEDGEMENTS

It gives me great pleasure in expressing my gratitude and thanks to all those people who have supported me and contributed in making this dissertation possible.

I express my deepest gratitude to Dr Asha Mary Abraham, my guide and mentor, for her constant guidance, support and encouragement during the course of completion of my dissertation. I am grateful to her for providing me the opportunity to work with this topic which has been a rich and rewarding experience.

I would also like to express my sincerest gratitude to Dr. Biju George, my co-guide for supporting me throughout my thesis with his knowledge, patience and constructive feedback.

I would also like to acknowledge the following people for their support and input at various stages of the work.

-The patients who willingly participated in the study

-Dr Rayaz Ahmed for helping in recruitment of patients into the study

-The nursing staff of Department of Hematology for helping me with sample collection at appropriate times

-Dr Rajesh Kannangai for his thoughtful and detailed comments and helping me with designing of primers for the study

-Mr Santosh Kumar, Mr Jaiprasath Sachithanandhan, Mrs Saranya Vijayakumar, Mrs Renita Ruth, Ms. Veena V Ramalingam and Mr K Anand for the technical aspects of the PCR and processing of samples

-The Institutional Review Board, Department of Clinical Virology and Department of Hematology for funding the study

-Dr V Balaji for his constant support and thoughtful concern

-My colleagues for their cheerful motivation, care and precious friendship

-My family for their constant prayers

Above all, to God who made it all possible.

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INTRODUCTION

Introduction

Human cytomegalovirus (HCMV) is one of the members of the *Herpesviridae* family and is a very common cause of human infection. The seroprevalence for the disease ranges from 50% among adults in United States to 90% among children of 4 years of age in India (1,2). However, the virus remains in equilibrium with the host immune system and disease manifestations are very rare in a healthy individual infected with the virus. Serious morbidity and mortality is seen in individuals with immature immunity such as neonates, and in individuals with compromised immunity such as solid organ and bone marrow transparent recipients (3).

Bone marrow transplantation is one of the treatment modalities for malignancies or abnormalities of the lymphohematopoietic system (4). Bone marrow transplant patients undergo immunosuppressive therapies to increase their chances of successful engraftment which leads to increased risk of developing opportunistic infections. Cytomegalovirus (CMV) infections form the major viral opportunistic infection in bone marrow transplant patients that manifest at 40-50 days after transplantation (5). After engraftment, the rate of incidence of active CMV infection is approximately 60–70% in CMV seropositive patients and CMV seronegative recipients' with grafts from seropositive donors. If no preventive measures are taken, approximately 20–30% of these patients progress to CMV disease (6). Since most of the patients already have a latent infection with cytomegalovirus even before the transplantation, they remain at risk of reactivation of the infection and thereby manifesting the disease. Although patients can be treated with the

antiviral drug ganciclovir, the prognosis for patients who have already manifested symptoms of the disease, even with treatment, is poor (7,8). One approach to the problem is by initiating pre-emptive therapy for which the patient is started on ganciclovir when there are indications of an impending viral infection. The importance of starting preemptive therapy at the most appropriate time cannot be overemphasised as initiation of therapy too early can lead to unnecessary exposure of the patient to ganciclovir, a bone marrow toxic drug, while delay in initiation of therapy can lead to non-response to therapy.

One of the indicators of an impending infection is CMV DNA levels in the blood of the patient (9). CMV DNA monitoring provides several advantages over the conventional method of quantitating pp65 antigenemia, such as faster turn over time, less hands-on work with more convenient work techniques and minimal inter- observer variability in experienced hands (10). CMV DNA monitoring was also found to be a more accurate method for detection of CMV levels than monitoring pp65 antigenemia (11). However no consensus has been reached on the CMV DNA threshold level at which treatment should be initiated. There is wide variation in the cutoff levels from centre to centre as different methods of CMV DNA quantitation are employed in each centre. Moreover, the geographical variations in the CMV seroprevalence rates also call for an individualized estimation of threshold levels.

Another approach to determining the onset of CMV disease is quantitation of CMV mRNA as it has been found to be a sensitive indicator of viral replication (12). Detection

of CMV transcripts in peripheral blood indicates that the virus is undergoing replication, and hence, it is likely that the patient has CMV disease.

The aim of this study was to measure the CMV viremia levels in blood by quantitating both CMV DNA and CMV Immediate Early (IE) - mRNA and comparing their levels to determine an appropriate threshold for initiation of pre-emptive therapy.

AIMS AND OBJECTIVES

AIM AND OBJECTIVES

AIM: To study the kinetics of CMV viremia in bone marrow transplant patients (BMT) undergoing CMV reactivation in the early post- transplant period.

OBJECTIVES:

1. To evaluate the kinetics of CMV DNA levels in BMT patients who reactivate CMV within the first 12 weeks post- transplant (by serial monitoring with real-time PCR).

2. To quantitate the CMV IE-mRNA levels in blood samples of BMT patients using real-time reverse transcriptase PCR (RT-qPCR) in the post-transplant period.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

1. Human cytomegalovirus

1.1 CMV infection

Primary human CMV infection is usually asymptomatic in an immunocompetent individual. Infection can also result in a mononucleosis syndrome, with fever, myalgia, lymphadenopathy, and hepatomegaly (13,14). However, CMV is capable of causing serious morbidity and mortality in neonates whose immune system is still immature. It is the leading infectious cause of neurological abnormalities and deafness in children, who were infected with the virus *in utero* (15,16). Another group of individuals who develop severe manifestations with CMV infection are the immunocompromised, such as people infected with human immunodeficiency virus and transplant recipients (17).

1.2 HCMV infection in haematopoeitic stem cell transplant patients

CMV infections form the major viral opportunistic infection in bone marrow transplant patients that manifest at 40-50 days after transplantation (5).The most common manifestations of the disease are pneumonitis and gastrointestinal disease (colitis) (18). After engraftment, the rate of incidence of active CMV infection is approximately 60–70% in CMV-seropositive patients or CMV-seronegative recipients who receive transplants from a seropositive donor. If no preventive measures are taken, approximately 20–30% of these patients progress to CMV disease (6).

2. Properties of HCMV:

2.1 Classification and taxonomy

CMV is referred to officially as Human herpesvirus 5, and belongs to the *Herpesviridae* family. Herpes viruses are classified into alpha, beta and gamma types based on their biological characteristics, genomic organization, tissue tropism, and amino acid identities (19). CMV along with Human herpesvirus 6 and 7 are classified as betaherpesviridae because of their long replicative cycle and restricted host range. The other members of the family include the human pathogens Human herpesvirus 1, 2 and 3 (with the common names of herpes simplex types 1 and 2 and varicella-zoster virus) which belong to alphaherpesviridae while Human herpesvirus 4 (Epstein-Barr virus) and Human herpesvirus 8 (Kaposi sarcoma-associated herpesvirus) belong to the gammaherpesviridae. The alphaherpesviridae members are characterised by their short cell cycle along with cytolytic properties. The gammaherpesviridae show variable cell cycles and exhibit a tropism for lymphoid tissue.

CMV was discovered in 1956 and was so named for the enlargement of the host cells after infection with the virus (20). The characteristic enlarged cells along with the inclusion bodies were observed by scientists even before the discovery of the virus. In 1881, Ribbert visualized the enlarged cells in kidney and parotid gland tissue sections of children. Jesionek and Kiolemenoglou, who had also made the same observation, had come to the conclusion that the intracytoplasmic inclusions could be protozoa. In 1932, Wyatt suggested the name “generalized cytomegalic inclusion disease (CID)” for

children affected by a congenital disease with presenting clinical signs of petechiae, intracerebral calcifications and hepatosplenomegaly. In 1956 Wellers, Smith and Rowe were independently able to grow and isolate the virus on human cell lines. In the same year, the virus was given the name cytomegalovirus by Thomas Wellers (21).

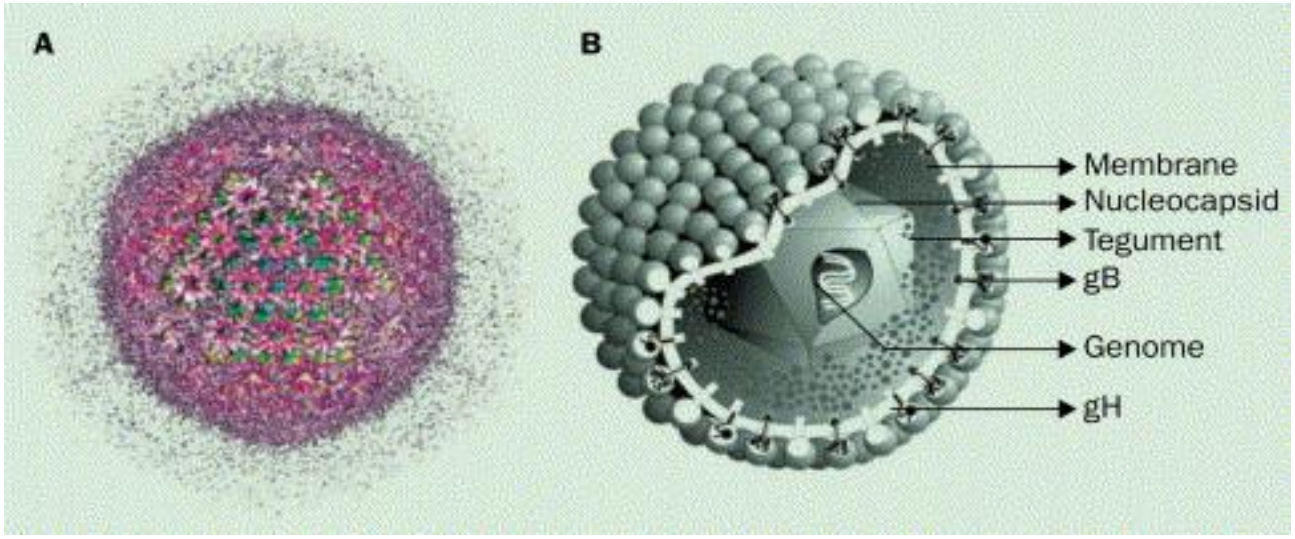
2.2 Structure of CMV

CMV is a double stranded DNA virus falling in Class 1 of the Baltimore classification (22). The virus has the largest genome among the herpes viruses with approximately 235 kilo base pairs encoding for 165 genes (23). The DNA is enclosed in an icosahedral capsid of 162 capsomers with a diameter of 110 – 125 nm. The nucleocapsid is composed of five herpesvirus core proteins (24). Outer to the nucleocapsid is a protein covering also called the tegument or matrix. The tegument contains the majority of the viral proteins along with some amount of cellular and viral RNA. The proteins present in the tegument play a role in maintaining the structure of the virus, aiding in its disassembly and assembly inside the host cell and also help in regulating the host immune response in an infection (24).

Mature virions have a diameter of approximately 200 – 300 nanometers and are completely enclosed in a lipid bilayer envelope that is derived from the host cell endoplasmic reticulum (ER)-Golgi intermediate compartment (ERGIC). The envelope contains approximately 20 virus-encoded glycoproteins that play a major role in cell attachment and penetration (24). Virion envelope glycoprotein B (gB) is responsible for

cell attachment and penetration while glycoprotein H (gH) is necessary for fusion of viral envelope with host cell membrane (24).

Structure of HCMV



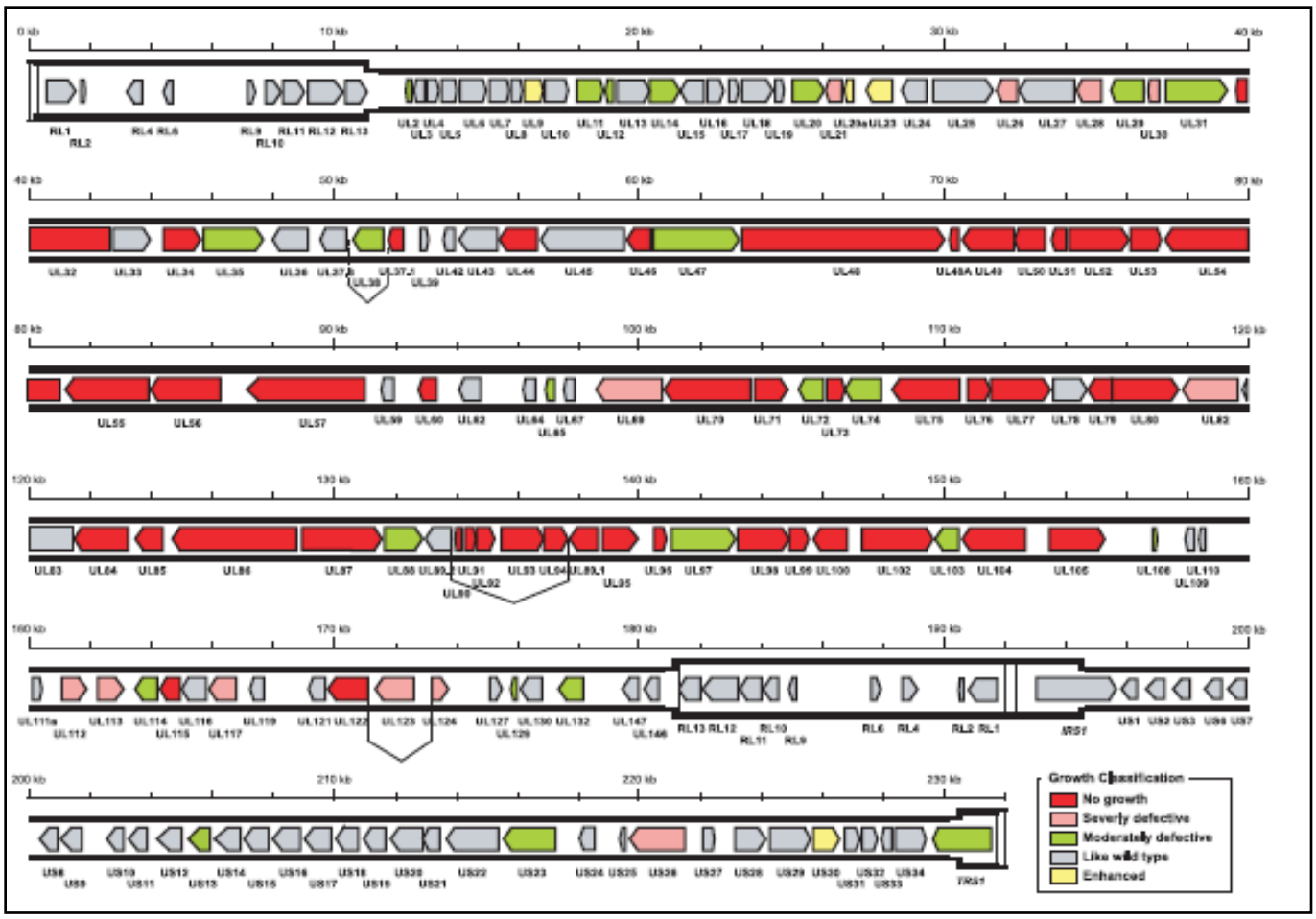
2.3 Viral Genome

The CMV genome has the distinction of being the largest genome among the herpes viruses infecting humans. It is a linear double stranded DNA molecule comprising 236 kilo base pairs. The genome is arranged into a unique long (U_L) and a unique short (U_S) sequence flanked by terminal and inverted repeats. The terminal repeat long (TRL) and the internal repeat long (IRL) flank the U_L region while the internal repeat short (IRS) and the terminal repeat short (TRS) flank the U_S region (25).

The genome encodes for approximately 166 genes including a single oriLyt and two transcriptional enhancers, one within the U_L and one within the U_S region (24). The

relatively high number of genes results in a crowded genome and the virus compensates for this bulky gene size by incorporating complex transcriptional processes. Some of them are described below:

a. Multiple transcripts that have the same initiation point and promoter but different termination points (26).
b. Multiple transcripts that have the same terminal points with the benefit of common poly-A signal and poly–A site but with different initiation sites (26).
c. Transcripts that can undergo complex differential splicing by removing differing intron regions to produce multiple final products (26).
d. Sense and antisense transcript pairs that are generated from complementary strands when read in opposite directions (26).



Schematic representation of HCMV genomic organization and open reading frames. Courtesy : Dunn W, Chou C, Li H, Hal R. Functional profiling of human cytomegalovirus genome. *Proc Natl Acad Sci U S A.*2003 Nov 25

2.3.1 Structure of the immediate early gene

The immediate early gene is the first gene to be expressed during productive primary infection and also in reactivation after a latent infection (24). The major immediate early genes are located at the UL122-123 region of the CMV genome. Alternative splicing of transcripts has been extensively employed by the virus at this site and this generates a

wide variety of transcripts some of which do not yet have any functions assigned to them to date. The UL123 region encodes for 4 exons and the most important product from this region is the 72-kDa major IE protein, IE72. Other minor transcripts derived from differential splicing of the same produce the proteins IE19, IE17.5 and IE9.The IE2 is represented immediately after the IE1 region at UL122. The major protein products from this region are IE86 followed by IE18 and IE55 (27).

2.4 Viral replication

The virus particles can be detected in various body fluids such as urine, saliva and breast milk during an active infection as well as during recovery from an infection indicating that the virus is capable of division in a variety of host cells. However, studies on the viral replication and other features are conducted in viruses that are passaged on fibroblasts (24). The virus interacts with host cell surface heparan sulphate and binds to it, following which the viral envelope fuses with the cellular membrane. This releases the nucleocapsid into the cellular cytoplasm (28). The cellular cytoskeleton then translocates the nucleocapsid to the nuclear membrane where interaction with the nuclear pores releases the viral DNA into the nucleus of the cell (29).

During replication, there is a highly coordinated expression of genes in the virus with the immediate early, delayed early and late proteins generated in sequence. This temporal cascade of viral gene expression is initiated with the transcription of immediate early genes promptly after viral entry as it does not depend on expression of any other viral gene. The immediate early proteins have profound effects on the host cells by

suppressing pathways that initiate interferon activation and disintegrating the major histocompatibility complex (MHC)-1 antigen presentation. This leads to some protection of the infected cells from the deleterious effects of cell mediated immune response of the host. These result in a conducive environment for the virus to replicate and the subsequent expression of the delayed early and late genes (30).

The delayed early proteins are reponsible for viral DNA replication and the late genes code for viral structural proteins. Viral DNA synthesis is initiated in productive infection of a cell with a gradual increase in DNA synthesis. Immediate early transactivators initiate transcriptional activation of orilytA region that is responsible for DNA replication. Viral DNA undergoing replication circularizes and replication follows a rolling circle mechanism. The proteins responsible for DNA synthesis are segregated in regions of the cellular nucleus called replication compartments which are seen as the classic intranuclear inclusions of CMV infected cells (31).

Viral DNA encapsidation occurs in the nucleus with a procapsid shell formed from five herpesvirus conserved proteins. Encapsidation of DNA and maturation of procapsid to nucleocapsid is enabled by a number of proteins, of which the most important is the maturational protease, also called assemblin (24).

The most popular theory regarding egress and release of virion particles is the envelopment/deenvelopment/reenvelopment process (32). As the nucleocapsid matures, it is released from the nucleus into the cytoplasm. During this process, a primary envelopment occurs at the inner nuclear membrane followed by a deenvelopment at the

outer nuclear membrane. During its course through the cytoplasm, the nucleocapsid acquires the proteins that form the tegument. The secondary envelopment occurs at the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) membranes (33). After final envelopment, the virion is incorporated into a vesicle and fusion of this exocytic vesicle with the plasma membrane releases the progeny virus into the extracellular space. The distinctive cytoplasmic inclusion body that is seen in an infected cell is the expanded endoplasmic reticulum and Golgi complex within which the nucleocapsids accumulate before their egress from the cell. This inclusion deforms the host cell nucleus giving it a kidney bean appearance, while the entire cell assumes an owl's eye appearance on visualization under the light microscope (24).

2.5 Latency and reactivation

CMV is capable of persisting in a latent form in an immunocompetent host following which periodic reactivation occurs under conditions are favorable for virus replication. The exact site of latency of the virus is not definitively known. However some evidence of presence of genomic viral DNA in cells of myeloid lineage suggests that they could harbor the virus during periods of latency (34). The cell lineages implicated are the monocytes/macrophages (35), lymphocytes (36), $CD34^+$ bone marrow cells (37), immature dendritic cells (38) and endothelial cells (39). Viral DNA is present in very low copy numbers in such cells, (2 to 13 copies per cell) making it difficult to detect the virus in a state of latency (40). The latency state is maintained by the transcriptional repression of the major Immediate Early promoter (41). Immunosuppression, inflammation,

infection and stress can trigger reactivation of CMV (42). Chemical mediators like Tumor necrosis factor α, proinflammatory prostaglandins and stress catecholamines can initiate transcription of CMV immediate early genes via cellular mediators, thereby promoting viral replication (43–45).

2.6 Immune responses to the virus

2.6.1 Innate immunity

The innate immune system plays a vital role in the body's defense against cytomegalovirus. Stimulation of toll like receptors (TLRs) by the antigens such as gB/ gH present on the viral envelope leads to production of inflammatory cytokines, generation of alpha/beta interferon by dendritic cells and macrophages, and upregulation of co-stimulatory molecules like CD80 and CD86 that trigger adaptive immunity (46,47). It has been demonstrated that natural killer cells play a major role in recovery from CMV infection in renal transplant patients (48).

2.6.2 Adaptive immunity

2.6.2.1 Humoral immunity

Neutralizing antibodies produced against the virus restrict its dissemination in the human body and reduce the severity of the illness in susceptible individuals. Most neutralizing antibodies are produced against conformational epitopes formed by the gH/gL/UL128-131A complex that mediates entry of the virus into various host cells (49,50). In humans,

transfer of anti-CMV antibodies from mother to new born infant protects the neonate from infection with the virus (51).

2.6.2.2 T-cell mediated immune responses

Cell mediated immunity is the essential arm of immunity against CMV; severity of illness in patients with compromised cellular immunity adding further evidence to this. However, even though the various components of cellular immunity ($CD8^+$ T cells and $CD4^+$ T cells) are able to contain the virus, it not sufficient to completely eradicate the virus from the body.

Studies conducted in mice with selective depletion of lymphocyte subsets have revealed that $CD8^+$ T lymphocytes play the major role in immunity mounted against murine cytomegalovirus (MCMV). An integral role of $CD8^+$ T cells in cellular immunity in bone marrow transplant patients was demonstrated by studies conducted by Riddell *et al* in 1992 and Walter and colleagues in 1995. They successfully transferred CMV-specific $CD8^+$ T cells to bone marrow transplant patients and observed that cellular immunity against CMV was effectively restored with no significant toxicity generated against the cells (52,53). UL123 (IE1), UL 122 (IE2) and UL83 (pp65) are the most immunodominant regions, to which the $CD8^+$ T cells mount an active response. Approximately 10% of $CD8^+$ T cells in the peripheral blood of healthy virus carriers are dedicated as anti CMV response. This subset expands and accumulates to form 40% of the $CD8^+$T cell pool in the elderly age group and this phenomenon has been termed "memory inflation" (54). This clonal expansion of cells may contribute to the immune

senescence in old age where the subset of naïve T cells is less compared to the younger age group (55). It also hinders the response to other pathogens and maybe a cofactor that enhances progression to AIDS in HIV co-infected individuals (56).

$CD4^+$ T cell response is also essential for generating protective immunity against CMV. Relatively, 9.1% of the total $CD4^+$ T cell population in an adult is primed to respond to CMV and the major immunodominant region for this subset of cells is the viral gB (57). $CD4^+$ T cells provide immunity by sustaining the antibody production, maintaining and reconstituting $CD8^+$ T cells and killing virus-infected cells by releasing enzymes like granzyme B (58).

2.7 Immune evasion by CMV

Although the immune system of an immunocompetent person is able to protect them from active infection by CMV, it is not adept at clearing the virus from the body or eradicating the state of viral latency. This characteristic exhibited by the virus may be due to various immune evasion tactics employed; these properties have been studied in murine cytomegalovirus and mouse models (59).

2.7.1Effect on Natural Killer cells

Viral UL40 protein enhances expression of HLA-E on the cell surface and this in turn inhibits NK cells (60,61). The virus produces UL18 which is structurally similar to the MHC Class 1 receptor that inhibits NK cell activity; UL18 also has higher affinity for the inhibitory NK cell receptor, $LILRB^+$ (62,63). In addition, UL83, UL141, UL16, UL112-1

and UL142 have been proven to cause down-regulation of the NK cell signaling pathways, thereby rendering them immunologically incompetent (64–68).

2.7.2 Effect on $CD8^+$ T Cells

Activation of $CD8^+$ T Cells is down-regulated by CMV by reducing the expression of MHC Class 1 on the surface of infected cells. This is achieved by the highly coordinated expression and action of viral proteins after infection. Viral gene products of UL2 and UL11 degrade the heavy chains of newly synthesized MHC Class 1 proteins (69). MHC Class 1 peptide complexes are retained in the endoplasmic reticulum by US3 of the virus and peptide translocation of the same is inhibited by US6 (70,71).

2.7.3 Effect on $CD4^+$ T Cells

Similar to the mechanism of MHC Class 1 downregulation, MHC Class 2 is suppressed in order to curb the immunogenicity and activation of CD4+ T Cells. US2 of the virus reverts HLA-DRα and HLA-DMα to the cytoplasm where it is degraded (72). In addition, it has been demonstrated that the protein transcribed by UL111 is structurally similar to the inflammatory cytokine IL10 (and thus named cmvLA IL10). This protein plays multiple roles in down-regulating the MHC Class 1 and 2 expression, suppressing peripheral blood monocytes, and interrupting the production of inflammatory cytokines (73–75).

In addition, infected cells express a cell surface glycoprotein which acts as an Fc receptor. This causes nonspecific binding of Fc portion of immunoglobulins to the cell surface, thus masking the infected cell from the host's immune system.

2.8 Pathology and clinical presentations of CMV infections

The spectrum of clinical presentation of CMV infections is varied and depends mainly on the age and immune status of the individual.

2.8.1 Congenital and perinatal infections

A mother acquiring a primary infection or undergoing reactivation of latent infection during pregnancy can transmit the virus to the fetus via the placental system. Congenital infection can cause death of the fetus *in utero* in approximately 20% of cases. In cytomegalic inclusion disease of newborns, the central nervous system and reticuloendothelial systems are the most severely affected. The major manifestations include intrauterine growth retardation, retinitis, microcephaly, thrombocytopenia, jaundice and hepatosplenomegaly. Around 95% of children born to mothers who have a recurrent infection with the virus are asymptomatic, with 7% to 25% presenting late in life with deafness and learning deficiencies (76).

2.8.2 Healthy adolescents and adults

Primary infection with CMV in immunocompetent adults is usually asymptomatic. A small proportion of individuals may present with symptoms resembling infectious mononucleosis which rarely requires therapy since most spontaneously recover (77).

However, there have been case reports of immunocompetent individuals with primary CMV infection presenting with end-organ disease such as hepatitis, meningitis, encephalitis, nerve palsies and Guillian-Barre syndrome (78,79).

2.8.3 Immunocompromised hosts

2.8.3.1 HIV infected individuals

CMV posed a significant threat to persons infected with HIV before the introduction of highly active anti-retroviral therapy (HAART). Patients with a $CD4^+$ count of 100 and below were most at risk of infection with CMV. However with the introduction of HAART, the incidence of CMV disease has drastically dropped in this population (80).

The most common manifestation of disease in these individuals is hemorrhagic retinitis with necrosis. Some other presentations include esophagitis, gastritis, enterocolitis, pneumonitis, hepatitis and immune recovery vitritis with posterior segment inflammation seen in patients who are started on HAART (81).

2.8.3.2 Solid organ transplant patients

CMV is a one of the major pathogens causing considerable damage in the post- transplant period in individuals who have received a solid organ transplant. More than 50% in this group develop symptoms of the disease with features such as febrile neutropenia, pneumonitis, enterocolitis, gastritis, hepatitis, retinitis and other end organ disease (82,83). CMV infection can also cause graft rejection (84–86), renal artery stenosis in renal transplant patients (87), bronchiolitis obliterans in lung transplant patients and

rapidly progressing coronary artery stenosis in patients with heart transplant (88,89). CMV infection has also been implicated in the increased predisposition to other opportunistic bacterial and fungal pathogens in this group (90).

2.8.3.3 Hematopoietic stem cell transplant patients

The prolonged immunodeficiency required in this group of individuals puts them at risk of reactivation and primary symptomatic infection with CMV. The details of the infections are discussed below.

2.9 Epidemiology of CMV infections

Although CMV infections are common throughout the world, geographical variations in seroprevalence do exist. Seroprevalence based on anti-CMV IgG antibodies also depends on the age group, socioeconomic status, and parity (91).

Several studies conducted in women of the reproductive age group around the world reveals high seroprevalence rates among women in South America, Africa and Asia, with the lowest rates recorded in Western Europe and United States of America. A study conducted by Sheevani *et al* in the northern states of India found statistically significant difference in the seroprevalence between women from rural compared to urban areas. They have reported a higher incidence of circulating antibodies in women from rural areas, living in poor socioeconomic conditions and poor hygiene. Another observation was the higher seroprevalence noted in the women of higher parity as compared to nulliparous women. This could be attributed to child-to-mother transmission (92).An

Indian study conducted by Venkitaraman *et al* in South India has shown that 90% of children are seropositive for CMV by the time they reach 4 years of age (2). The higher seroprevalence in the low socioeconomic status may be attributed to higher population density arising from the higher number of children per family (since crowded living conditions allow better transmission of the virus easier) and the low standards of hygiene.

3. Hematopoietic stem cell transplantation (HSCT)

3.1 Introduction to bone marrow transplantation

Bone marrow transplantation (BMT) is one of the treatment modalities for malignancies or abnormalities of the lymphohematopoietic system and has evolved to become the standard of care in many hematological malignancies. Conditions where BMT plays a crucial role in treatment are diseases of bone marrow inadequacy like aplastic anemia, malignancies (leukemias), lymphomas, hemoglobinopathies, multiple myeloma, severe immunodeficiency syndromes, inborn errors of metabolism (e.g.chronic granulomatous disease) and high-risk solid organ disease like neuroblastoma (18). The procedure involves the transfer of hematopoietic cells from a donor to a recipient who has undergone a conditioning regimen that enables them to accept the transplanted cells.

3.2 Types of HSCT

HSCTs can be classified into allogeneic and autologous transplants based on the source of the donor cells. In autologous transplants, the cells are harvested from the patient, which are later re-infused into the same patient after high dose myeloablative therapy, in

preparation for the transplant. Syngeneic transplants are very similar to autologous transplants in that an HLA identical twin is the donor. Such transplants are most commonly used in diseases like breast cancer, Hodgkins lymphoma and non Hodgkins lymphoma (93). The advantages of this procedure are the low risk of development of graft versus host Disease (GVHD) and faster immunological recovery. However, they are at risk of a relapse from the graft if tumor cells contaminate the stem cell transplant (94).

In allogeneic transplants, the cells are harvested from a donor other than the transplant recipient. This procedure is used in conditions like aplastic anemia and chronic myelogenous leukemia. Sources of stem cells for such candidates include HLA matched siblings, family members or unrelated members who can be accessed through registry organizations such as (DATRI) that maintain computerized databases of HLA types of voluntary donors. Compared to autologous transplants, these patients are at a higher risk of GVHD, graft rejection and delayed immunological recovery (95).

3.3 Immune system and opportunistic infections before and after HSCT

3.3.1 Immunity during the conditioning regimen

The normal process of hematopoiesis generates viable neutrophils, monocytes and macrophages and this process is destroyed during the conditioning regimen. Mucosal progenitor cells are wiped out leading to a temporary loss of mucosal barrier immunity. Hence, commensal bacteria and fungi that are lodged in the gastrointestinal tract, skin and other mucosae then become potential pathogens. T lymphocytes and B lymphocytes

(primed to antigen exposure from natural infections, vaccines and environmental antigens) and memory cells generated over the lifetime of the patient are entirely obliterated, rendering the patient immunologically defenseless.

3.3.2 Pre-engraftment, Engraftment and Post-engraftment

The pre-engraftment period is defined as the time period from the start of conditioning to 20-40 days in the immediate post-transplant period. The patient is at risk of bacterial infections with the depletion of neutrophils and obliteration of the mucosal barrier. Failure of neutrophil recovery by day 42 is considered to be graft failure (18). Engraftment is defined as the time at which a patient can maintain a constant absolute neutrophil count of more than 500/mm^3 and a sustained platelet count of more than 20,000 cells/mm^3, for more than at least three consecutive days without transfusions (96). Engraftment occurs at around the 22^{nd} day in the post-transplant period for unrelated allogeneic transplants, and almost always within a range of 6 to 84 days (97). The post-engraftment period is marked by the recovery of neutrophils lasting upto the 100^{th} day after transplantation. The late risk period is taken as the time period after day 100 till the time the patient regains normal immunity, which can last from 18-36 months after transplantation (98).

After neutrophil recovery, monocytes and Natural Killer (NK) cells recover followed by platelets and red blood cells. Lymphocytes are the last set of cells to recover, beginning with NK cells, followed by $CD8^+$ T cells. B lymphocytes recover later while $CD4^+$ T cells take the longest time to recuperate.

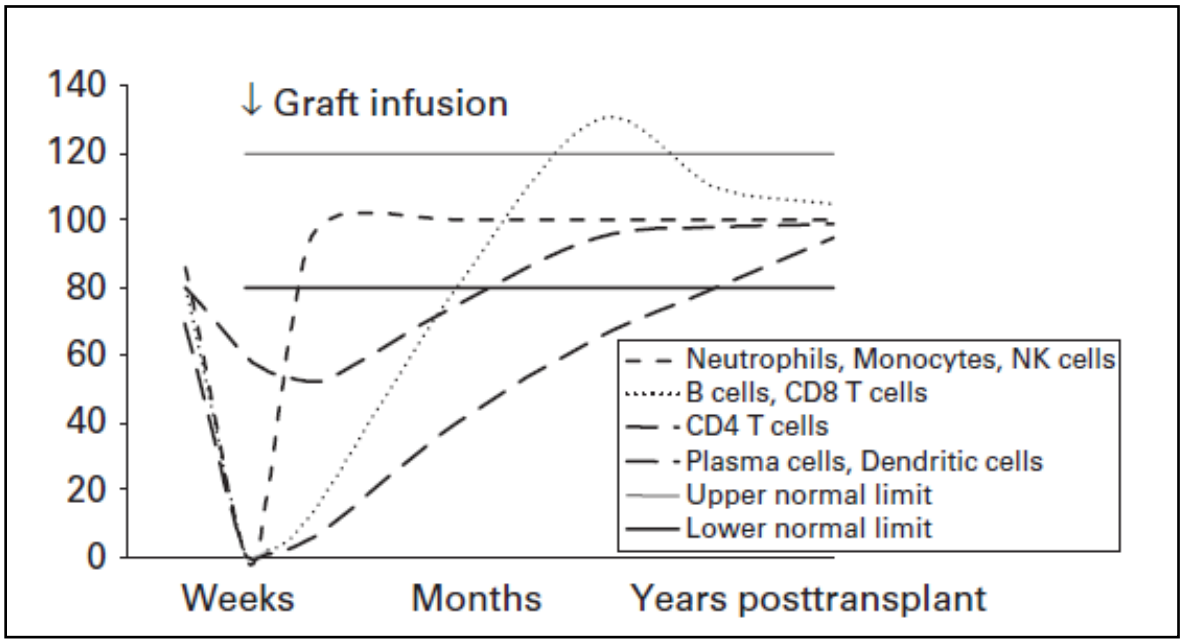
Regeneration of lymphocytes can follow two distinct pathways and the process is inefficient, making the recovery process slow. In one pathway, lymphocytes, mainly NK cells and B lymphocytes, regenerate from bone marrow lymphoid progenitor cells, thereby creating a naïve immune system similar to that of young children. Also, the specialized marrow microenvironment called the 'bursal equivalent' (essential for B cell regeneration) is highly sensitive to the toxic effects of the conditioning regimen, GVHD or treatment undertaken for the same. Hence, B cells take longer to regenerate than NK cells (99).

Complete reconstitution of humoral immunity requires the generation of memory B cells. These cells are produced on re-exposure to either environmental antigens or vaccines. The entire process, which also requires the help of $CD4^+$ T cells, takes upto a year for full recovery. For these reasons, the patient remains susceptible to infections from encapsulated bacteria and viruses during the first year after transplantation, as the essential mode of defense against these organisms are neutralizing antibodies (99).

Regeneration of T cells occurs through an expansion of T cells present in the graft that responds to T cell deficiency *in vivo.* This is a thymic independent pathway as opposed to the normal development of T cells in healthy individuals, and is termed 'homeostatic peripheral expansion'. A combination of factors drives this process, including increase in cytokines like IL-7 and IL-15, release of inflammatory cytokines during tissue damage during the preparative regimen, and exposure to viral antigens. Regeneration of $CD8^+$ T

cells is more efficient than that of $CD4^+$ T cells, resulting in a higher CD8/CD4 ratio for several months after transplantation (99).

Although it is imperative that all HSCT patients undergo immunosuppression for successful engraftment, the severity of the immunosuppression and recovery of the immune system post-transplant depend on a number of factors. Some of these factors include the development of GVHD, recipient factors like age, co-morbidities, and exposure to infectious agents (99).



Approximate immune cell counts before and after myeloablative HSCT. Courtesy:Mackall C *et al*. Background to hematopoietic stem cell transplantation, including post transplant immune recovery. Bone Marrow Transplantation.2009; 44: 457–462

3.3.3 Infections following HSCT

Opportunistic infections affecting HSCT patients can be classified into three phases depending on the immune status of the patient

3.3.3.1 Phase 1: Pre-engraftment phase

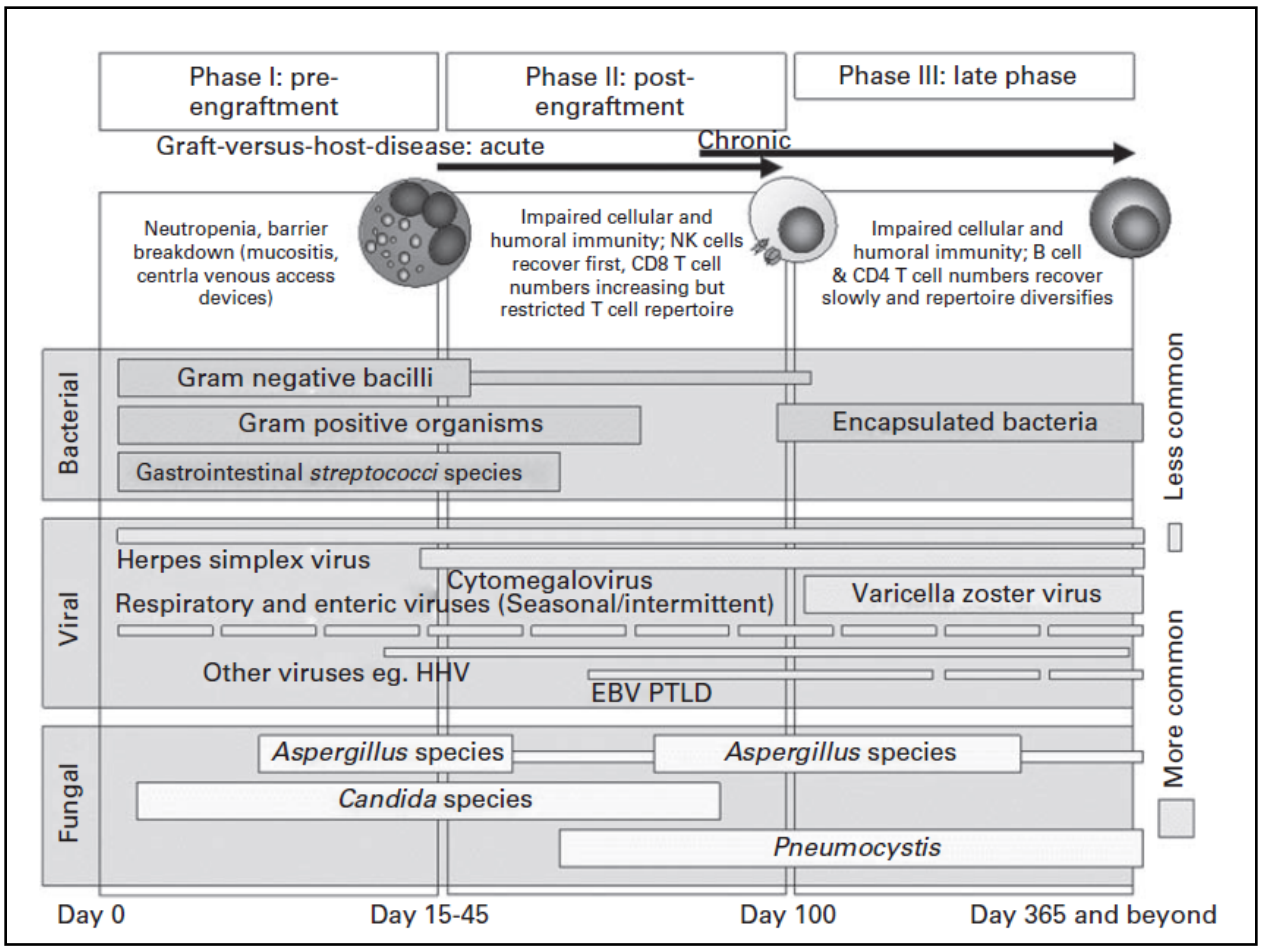
The factors that predispose to infections in this period are prolonged neutropenia, mucosal barrier breaks and the intermittent use of vascular catheters for patient care. The most common pathogens seen in this phase are bacterial organisms followed by *Candida* and *Aspergillus* spp (96).

3.3.3.2 Phase 2: Post-engraftment phase

During this phase, the patient has deficient cell mediated immunity resulting in a higher risk for infections due to herpes viruses like cytomegalovirus, *Pneumocystis carinii* and *Aspergillus* spp (96).

3.3.3.3 Phase 3: Late phase

During this phase, the patient has deficient cell mediated immunity and humoral immunity along with impaired reticuloendothelial cell functions. The predominant pathogens during this phase are cytomegalovirus, varicella zoster virus, Epstein Barr virus, community acquired respiratory viruses and encapsulated bacteria like *Haemophilus influenzae* and *Streptococcus pneumoniae* (96).



Phases of immune system recovery and opportunistic infections among HSCT patients. Courtesy:Mackall C *et al*. Background to hematopoietic stem cell transplantation, including post transplant immune recovery. Bone Marrow Transplantation.2009; 44: 457–462

4. CMV infections in HSCT recipients

4.1 Factors affecting development of CMV infection in HSCT patients

4.1.1 CMV Serological status of recipient and donor

CMV serological status of both the recipient and donor influence the development of CMV disease in the post-transplantation period. If the recipient is seronegative, care should be taken to select a donor who is also seronegative. Transplant-related mortality is lowest when a CMV seronegative recipient receives stem cells from a donor who is also CMV seronegative (100). If the recipient is seropositive or if a seronegative recipient is infused with cells from a CMV seropositive donor, the risk of CMV disease reaches 60-70%. If no preventive measures are taken, approximately 20–30% of these patients progress to CMV disease (6).The higher risk of developing CMV disease with a donor positive/recipient negative situation may be attributed to the indirect immunomodulatory effects of CMV as these patients were shown to have a higher incidence of bacterial and viral diseases in the post-transplant period, as shown by Nichols *et al* (101).

The transplant related mortality outcome as a result of having a CMV seropositive donor has been controversial. Some studies have suggested that transplantation of CMV seropositive recipients with CMV seropositive unrelated donors had better outcomes with higher event-free survival rates and reduced transplant related mortality (102). A megafile analysis by Ljungman *et al* from data of the European Group for Blood and Marrow Transplantation (EBMT) registry provides results that corroborate this finding.

The study also concluded that patients who received grafts from a seronegative donor had an increased risk of death. One of the possible mechanisms suggested for this negative interaction is lack of transfer of CMV specific effector T lymphocytes (103). However this observation is debatable and the findings were not reproduced in other studies (104).

4.1.2 Effect of source of stem cells and CD34 selection

Studies have shown that transplants conducted with unrelated or mismatched related donors have a higher rate of development of CMV disease and transplant related mortality (105,106). Similarly, umbilical cord blood transplant recipients are at a higher risk of CMV disease if they are not started on appropriate antiviral prophylaxis (107). Reconstitution of CMV specific T cells occurs much faster with autologous HSCT. Even though these patients may show a short-lived phase of viral replication, they rarely progress to CMV disease; there is thus no need to start antiviral therapy in such patients (108,109).

While CD34 depletion theoretically removes contaminating tumor cells from the graft, it is associated with higher rates of CMV disease in both allogeneic and autologous transplant patients undergoing myeloablative conditioning regimen (110).

4.1.3 Effect of immunosuppression in the post-transplantation period

An immunosuppressive regimen is administered post-transplantation to prevent rejection of the graft and avoid complications from development of GVHD. Treating patients with mycophenolate mofetil, high dose corticosteroids(>1mg/kg/day), Alemtuzumab(anti-

CD52 monoclonal antibody) (111), antithymocyte globulin and use of T cell-depleted allografts and autografts, are considered high risk factors for the development of CMV disease (112,113).

The severity of immunosuppression determines the time it takes for the immune system to recover, and hence it has an indirect effect on the replication dynamics of the virus. Hence, in severely immunocompromised recipients, the viral load doubles in 24 hours as shown by studies conducted by Emery *et al* (114).

4.2 Clinical manifestations of CMV disease

CMV disease can present as either early disease occurring within the first 100 days following transplantation or late disease manifesting after 100 days. The most common presentations of early disease are gastrointestinal disease affecting the gastrointestinal tract anywhere from the oesophagus to the colon, interstitial pneumonia affecting the lungs and hepatitis (6). During the late phase, CMV disease can manifest as CMV retinitis or affect the central nervous system (115,116). These manifestations can also be termed as the direct effects of CMV infection. Some of the definitions associated with CMV disease are:

CMV infection is defined as detection of the virus, viral proteins or nucleic acid in any body fluid or tissue specimen (117).

Primary CMV infection is defined as the detection of CMV in a previously CMV seronegative individual (117).

Recurrent infection is defined as new detection of CMV infection in a patient who has been previously documented to have infection and who has not had virus detected for at least 4 weeks of active surveillance. Recurrent infection can be a reactivation of latent virus (endogenous) or reinfection (exogenous) (117).

CMV disease is defined as detection of CMV by immunohistochemical analysis of biopsy specimens followed by clinical signs and symptoms, such as unexplained fever (> 38°C), leucopenia (white blood cells < 3.5×10^9/L) and/or thrombocytopenia (platelet count < 100×10^9/L), gastrointestinal symptoms, arthralgia, hepatitis, enteritis, retinitis, pneumonitis, colitis, oesophagitis and encephalitis (118).

The most serious manifestation of CMV disease is interstitial pneumonia and approximately half the patients with this complication succumb to it (119). It manifests as fever, nonproductive cough and hypoxia. Fever may not be present in patients who are subjected to high degrees of immunosuppression (120). CMV gastroenteritis presents as ulcers of the gastrointestinal tract which may extend into the submucosal layer.

Indirect effects of CMV infection in HSCT patients include graft rejection and superinfection with bacteria and fungi due to further weakening of the patient's immunity (101,121).

5. Diagnosis of CMV infection and disease

Patients selected for an HSCT should undergo serological evaluation of anti-CMV IgM and IgG antibodies to determine their serostatus. This is also applicable for the donor whose serostatus is a definite predictive indicator for the development of CMV disease in the post-transplant period. However the use of serological indicators for evaluation of CMV disease in the post transplant period is controversial. Although some clinicians do not deem serological indicators to be of any value in diagnosing CMV disease (120), there have been contradicting studies conducted on the efficacy of determination of rising titres of anti-CMV IgG as well as detection of anti-CMV IgA and IgM. These studies have pointed out that serological indicators may be used as a marker of CMV reactivation in the post transplant period especially in laboratory settings where PCR and antigenemia tests are unavailable (122,123).

In the event of CMV end organ disease, a diagnosis can be established by virus isolation and culture from samples representing the diseased organ, in situ DNA hybridization, direct fluorescent antibody tests or immunohistochemistry (117). Diagnosis of end organ disease like pneumonia and gastrointestinal disease by molecular techniques such as PCR for CMV DNA may be too sensitive and interpretation of results may not be conclusive as silent reactivation of the virus can happen in these tissues without causing disease. Hence PCR is not a useful test for diagnosis end organ disease and may be used only when suspecting CMV encephalitis, a rare entity (117,124)

5.1 Viral isolation and culture

CMV culture is done on fibroblast cell lines derives from human embryonic tissue, foreskin tissue and human foetal lung tissue. Patient samples such as urine and blood can be inoculated on these cell lines. The virus is detected by determining the cytopathic effect (CPE) which includes enlargement of the host cell, with intranuclear inclusions. The day of appearance of CPE varies with the virus titre in the sample. High titre samples may yield results in 24 – 48 hours. Culture results may be delayed for upto 6 weeks for low titre samples (125). Detection of the virus on cultures can be improved by using immunoflouresence techniques like employment of monoclonal antibodies against viral antigens. The turnaround time of viral culture can be enhanced by techniques such as shell vial culture on coverslips and centrifugation to enhance viral uptake on cell lines (126,127).

Quantitation of virus in culture can be achieved by plaque assay and titre determination (50% tissue culture infective dose or $TCID_{50}$). The plaque assay is performed by infecting fibroblast monolayers with serial dilutions of the specimen and overlaying this with a semisolid medium. The monolayers are incubated at 37^{o}Cand the reading is taken by counting the numbers of infectious plaques formed in each dilution under the microscope (128). For determination of $TCID_{50}$, fibroblasts monolayers are inoculated with serial dilutions of the specimen and readings taken after 3-6 weeks. The virus titre is estimated by determining the highest dilution that produces a cytopathic effect in 50% of inoculated cell cultures. Shell vial culture uses fibroblast cell culture monolayers on a coverslip and

centrifugation of sample onto the monolayer to enhance recovery rate and reduce the recovery time of cultures. Although viral cultures do not require sophisticated laboratory techniques or equipment, it is still a time consuming procedure with low sensitivity (129).

Detection of early antigen immunoflourescent foci (DEAFF) is a rapid culture based diagnostic assay that employs antibodies against viral antigen to detect viral growth on shell vial cultures after 16-24 hours of incubation (130,131). However, culture based techniques may not yield reliable results in transplant patients as drugs such as antibiotics, antifungals, cytotoxic agents and immunomodulators may be toxic to cell lines used for cultures (132).

5.2 pp65 antigenemia assay

The assay utilizes monoclonal antibodies directed against pp65(UL83) to detect and quantitate CMV infected peripheral blood polymorphonuclear leukocytes(PMNL) (133). The PMNLs are separated from the patient's blood sample using dextran and fixed onto slides using chemicals such as acetone, formaldehyde-Nonidet P-40 etc. The cells are counted and treated with monoclonal antibodies. The slides are then viewed using a flourescence microscope and the number of cells that flag positive are counted. This test does not require highly sophisticated laboratory facilities and yields faster results than cell culture. It has also been demonstrated in comparison studies that the levels of antigenemia correlate well with CMV DNA levels in plasma and leukocytes. However, the cell counting process is laborious and there may be inter observer variability in determining the result (129).

5.3 Molecular assays for quantification of CMV

5.3.1 Branched-DNA Signal amplification assay

The branched DNA (bDNA) signal amplification assay can be performed on blood, CSF and semen samples directly. For blood samples, leukocytes are separated using dextran and lysed using Proteinase K to release nucleic acid. The lysate is then allowed to react with CMV specific target probes bound to a 96 well microtitre plate for 16 – 18 hours. The bDNA amplifier molecules (that have approximately 45 sites for binding of alkaline phosphatase labeled probes) are then added. The signal is read using a luminometer after allowing the enzyme alkaline phosphatase to react with a chemiluminescent substrate. Quantification is done by comparison with a standard curve (134). There are only a few reports on the performance of this assay. As there is no amplification of DNA in this procedure, there is very little chance of cross contamination. However, the long incubation step increases the turnaround time for the results (129).

5.3.2 Hybrid capture CMV DNA assay

This test is performed on leukocytes separated from whole blood. After lysis, the released DNA is allowed to react with CMV specific RNA probes that are complimentary to approximately 17% of the viral genome. The RNA-DNA complex is then immobilized using antibodies specific to the complex which is then tagged with antibodies labeled with alkaline phosphatase and a chemiluminescent substrate (135). The advantages of the test include the lack of an amplification step or prolonged incubation. A newer second

generation assay also claims to have a lower limit of detection of 700 copies of genome per ml of whole blood. However, comparison studies with the antigenemia assay and quantitative DNA PCR assays show conflicting reports, with some assays concluding that the assay is inferior to both the other tests (136,137).

5.3.3 Quantitative CMV DNA PCR

The quantification of nucleic acids using PCR is based on the formula $Y = X (1+E)_n$ where Y stands for the amount of DNA obtained after amplification with PCR, X is the quantity of target DNA before amplification, E is the efficiency of the PCR reaction and n is the number of cycles of amplification. The quantity of amplified DNA is estimated by comparing it with a standard curve that is derived by amplification of known quantities of target DNA (129).

Real time PCR using Taqman probes quantitates the amount of DNA accumulating in each cycle at the same time as the amplification process (that is, in real time). This alleviates the need for post PCR processing and allows for earlier issue of reports. Taqman probes have sequences that are complimentary to the target DNA and contain a reporter dye and quencher dye. The proximity of the reporter dye and quencher prevents the reporter dye from emitting a signal. As the amplification proceeds, Taq polymerase cleaves the reporter dye from the probe, thus removing it physically away from the quencher molecule. The reporter dye thus emits a signal that is detected by the machine and with each cycle of amplification, the increase in intensity is measured to give an estimate of the final DNA concentration (138).

Real time PCR for CMV DNA can be carried out on whole blood, plasma, leukocytes separated from whole blood, CSF, and other samples. Systemic viral load can be estimated using whole blood, plasma or leukocytes. Since the virus is usually associated with the cellular fraction, quantitative PCR for DNA would be higher if performed on whole blood or leukocytes than plasma. However studies have established that the DNA estimates from that of plasma correlate well with that of whole blood or buffy coat (139).

5.3.4 Quantitative CMV mRNA estimation

Although CMV antigenemia testing and DNA detection are convenient methods of detection of the virus, a positive result need not indicate an active infection in the patient.. Detection of CMV transcripts in peripheral blood indicates that the virus is undergoing replication, and hence the possibility that the patient may have CMV disease cannot be ruled out. IE mRNA is detected within one hour of active infection of a cell and RNA of late structural proteins such as pp67 or pp150 can also be looked for (12). Using reverse transcriptase enzyme, mRNA is converted into complimentary DNA (cDNA) followed by amplification with PCR. However, the major concern of such a test was the contamination of the RNA extracts with CMV DNA which yielded false positive results. In order to circumvent this disadvantage, several techniques were employed such as treating the nucleic acid extract of the sample with DNase enzyme and designing primers flanking the intron region. However, even these techniques suffered setbacks when DNase failed to completely remove the contaminating DNA and some of the transcripts did not have any introns or splicing mechanisms. Nucleic acid sequence based

amplification was a novel technology introduced in 1991 in which RNA can be amplified even with contaminating DNA (140). The technology employs the use of the enzymes avian myeloblastosis reverse transcriptase, RNase H and T7 RNA polymerase. It is an isothermal amplification technique with amplification occurring at 41ºC. Contaminating DNA does not interfere in this process as the process of DNA amplification in this process is highly inefficient (141).

6. Treatment of CMV disease

Intravenous ganciclovir is the drug of choice for treatment of CMV disease (142). Ganciclovir is a nucleoside analogue and has to undergo phosphorylation by viral and cellular kinases for activation. After activation, ganciclovir triphosphate binds to viral DNA and causes premature termination of chain synthesis. It is also an inhibitor of viral DNA polymerase enzyme. Valganciclovir is the prodrug of ganciclovir and has tenfold higher oral bioavailability than ganciclovir (143). The disadvantages of using ganciclovir are the development of neutropenia and emergence of drug resistance (144,145). Patients undergoing treatment with ganciclovir should be carefully monitored for neutropenia with absolute neutrophil count checks, at least twice a week (146).

Foscarnet, a pyrophosphate analog, is the second line drug recommended in case resistance develops to ganciclovir. It acts by binding on the pyrophosphate binding site of CMV DNA polymerase and preventing its activity (143). The administration of foscarnet is associated with significant renal toxicity and electrolyte imbalances such as hypocalcemia, hypomagnesemia and hypophosphatemia (18). Hence the patient should

be prehydrated before administering the drug and continuously monitored for electrolyte imbalances.

CMV gastroenteritis is treated with ganciclovir or foscarnet alone. Duration and dosing of therapy depends on the extent of disease and depth of ulcers. Induction therapy can last for 2-3 weeks followed by several weeks of maintenance dosing. Since CMV pneumonia has higher mortality rates and is difficult to treat, a combination therapy of intravenous ganciclovir or foscarnet along with intravenous immunoglobulin is suggested (147).

Treatment of concurrent conditions such as GVHD and optimal supportive therapy with proton pump inhibitors is also critical in recovery.

7. Prevention of cytomegalovirus disease

7.1 Prevention of primary CMV infection

If a patient is seronegative for CMV before transplantation, care should be taken to prevent CMV infection both before and after transplantation. The patient should be transfused only with either blood products from CMV seronegative donors or leukocyte-depleted filtered blood products. In such patients, selecting a donor who is CMV seronegative is an important screening criterion and holds as much importance as finding an appropriate HLA match (120).

7.2 Prevention of CMV reactivation or infection

7.2.1 Choice of donor

The choice of an appropriate donor to be selected for a recipient who is CMV seronegative is debatable. Several studies have shown that transfusing such patients with cells from CMV seropositive donor is protective for the patient with better survival rates (102). Also it has been shown that patients receiving cells from CMV seronegative donors had increased rates of CMV reactivations and disease (148).

7.2.2 Antiviral chemoprophylaxis

Drugs such as high dose acyclovir, valacyclovir (which is a prodrug of acyclovir with better oral bioavailability) and ganciclovir have been administered as prophylactic agents in HSCT patients who are at high risk of developing CMV disease. Although prophylaxis with ganciclovir was highly effective in reducing incidence of CMV disease, it was associated with a number of other adverse events. Ganciclovir, being a potentially marrow toxic drug, can itself cause neutropenia which in turn predisposes the patient to other invasive bacterial and fungal infections (149,150). In order to prevent the neutropenia, growth factors can be administered to the patient. However, this adds to the cost of an already expensive procedure. Also the subset of patients, who may never develop the disease, would be given a marrow toxic drug unnecessarily.

7.2.3 Preemptive therapy and viral load monitoring

Another approach to the prevention of disease is by preemptive therapy, wherein ganciclovir therapy is instituted upon detection of increasing levels of CMV in blood(an indicator of active viral infection (9). The goal of initiating preemptive therapy is to prevent the development of disease in patients. Hence, the window period at which this therapy is initiated is crucial. Emery *et al* have shown in a study that the initial viral load and the rate of increase in viral load are important predictors of impending CMV infection (114).

CMV DNA detection by quantitative real time PCR is the most commonly used method used for monitoring CMV levels in the patient's blood. When compared to pp65 antigenemia, CMV DNA quantification by PCR offers the benefits of increased sensitivity for detection of reactivation of CMV, reduced time to perform the procedure, reliable results even with severe neutropenia in the early post-transplant period and ability to process large numbers of specimens at a time (10). DNAemia is also a more accurate indicator of actual viral replication *in vivo* than antigenemia (11). Preemptive therapy can be initiated when a certain viral load threshold is crossed or when the rate of increase of CMV DNAemia over time is significant, hence avoiding unnecessary treatment of patients who may not progress to disease (147). Although there are several studies from around the world that suggest DNA cutoff values at which pre-emptive therapy can be started, they cannot be used as standard cutoff values as they utilize different primer sets, target sequences and extraction and detection methods.

In a study conducted by Emery *et al*, the average doubling time of CMV was found to be only 1 to 2 days and that the *in vivo* replication dynamics depended on the degree of immunosuppression (114). Although the initial viral load is a good predictor for the risk of CMV disease, the viral load increases may also provide potential grounds for initiating preemptive therapy. When monitoring viral load, the variability inherent to the assay should be considered before setting a cutoff for initiation of therapy, to ensure only patients with "true increases" are given therapy. Since the coefficient of variation of most molecular assays that detect DNA approaches 30% near the limit of detection, only increases of more than $0.5\log_{10}$ rise should be considered an indicator for preemptive therapy (10,129,147).

MATERIALS AND METHODS

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Study design:

This is an observational study conducted in the patients undergoing hematopoietic stem cell transplantation in the Department of Hematology, Christian Medical College, Vellore and the diagnostic tests for CMV were conducted in the Department of Clinical Virology.

Study duration:

The study was conducted over a period of 17 months from April 2012 to August 2013

Recruitment of study subjects

Inclusion Criteria:

1. Patients undergoing bone marrow transplant at our centre
2. Patients who reactivate CMV within first 100 days following BMT

Exclusion Criteria:

1. Patients who do not consent to the study
2. Patients who reactivate beyond 100 days following BMT

Sample collection for CMV DNA PCR:

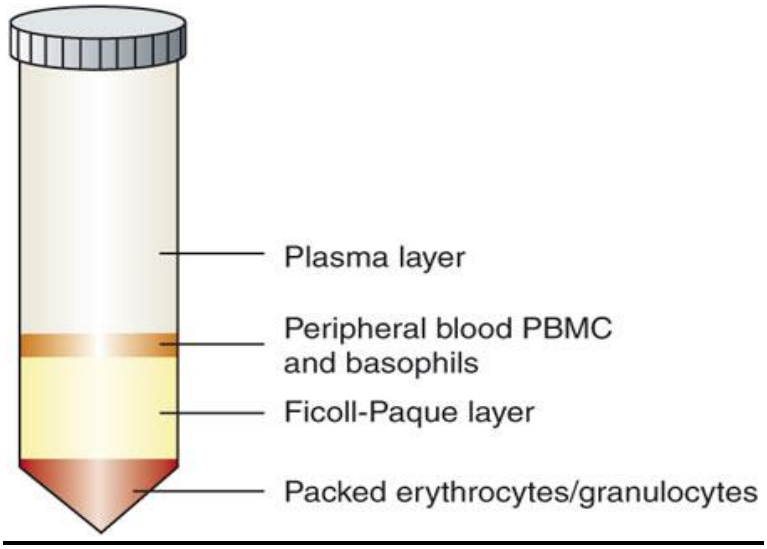
Patients were enrolled after taking appropriate consent. Blood samples were collected from the patients once a week for the first three weeks after transplantation till engraftment. Thereafter from week 4 to week 12, blood samples were taken biweekly. 5-6 ml of blood was collected in 6ml K_2EDTA BD vacutainer tubes (BD Biosciences). The samples were transported in ice to the Department of Clinical Virology. The samples were stored at -20ºC as two aliquots of 200µl and 1ml in 1.7ml micro centrifuge tubes.

Sample collection for CMV mRNA RT-PCR:

Quantitation of the IE-mRNA was performed only retrospectively after the patients reactivated for HCMV. Therefore there was the need to store these samples in RNA LATER solution (Qiagen, Hilden, Germany). However RNA LATER solution cannot be used for preservation of RNA in whole blood as the solution forms insoluble complexes with plasma. Hence the samples were stored as peripheral blood mononuclear cells (PBMCs), as RNA LATER can be used for the preservation of RNA in lymphocytes.

PBMCs were separated from whole blood using Ficoll-Paque Plus (GE healthcare, Uppsala, Sweden). Ficoll-Paque is based on the principle of density gradient separation. When whole blood is centrifuged with Ficoll-Paque the red blood cells aggregate at the bottom of the tube followed by the granulocytes. The Ficoll-Paque with a density of 1.099 forms a transparent layer above the granulocytes, thus separating the lymphocytes, monocytes and platelets. These three cells form a layer between the Ficoll-Paque and plasma. The cells are carefully retrieved and washed several times to yield pure PBMCs.

The PBMCs were stored in RNA LATER as two aliquots of 200µl at -80ºC in 1.7ml micro centrifuge tubes.

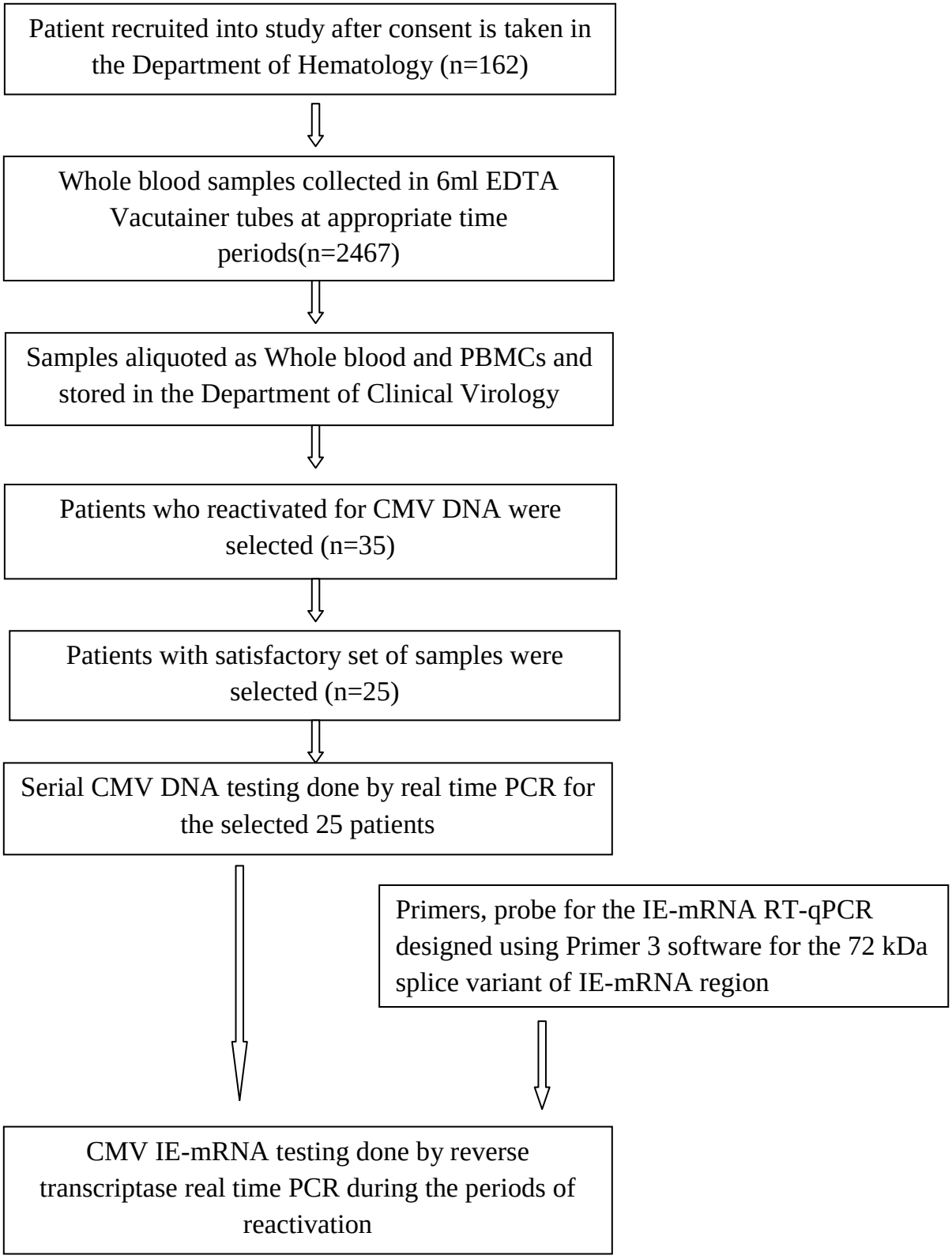


The different layers formed after centrifuging whole blood with Ficoll-Paque. Courtesy: Munoz et al. Highly purified selective isolation of eosinophils from human peripheral blood by negative immunomagnetic selection. *Nature Protocols* 2007

Schematic representation of time period of collection of samples

DNA	✓		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
WEEKS	**Pretransplant**	**Transplant**	**1**	**2**	**3**	**4a**	**4b**	**5a**	**5b**	**6a**	**6b**	**7a**	**7b**	**8a**	**8b**	**9a**	**9b**	**10a**	**10b**	**11a**	**11b**	**12a**	**12b**	
RNA						✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

STUDY ALGORITHM



Separation of PBMC using Ficoll-Paque:

Materials required:

15 ml centrifuge tubes with conical bottom (Tarson products. Kolkata, India)

Ficoll-Paque plus (GE healthcare, Uppsala, Sweden)

Phosphate buffer solution (PBS) [pH: 7.2 – 7.4]

1000µl long reach pipette tips

Centrifuge with swing buckets

Procedure:

1. Label five centrifuge tubes with the sample number.

2. Mix the blood sample well and pipette 3ml of whole blood into the centrifuge tube. Dilute the blood with 6ml PBS. Mix well to dilute the sample.

3. Pipette out 3ml of Ficoll-Paque into 3 separate centrifuge tubes.

4. Carefully layer 3ml of the diluted blood sample onto the Ficoll-Paque in the three separate centrifuge tubes using the 1000µl reach pipette tips.

5. Centrifuge the centrifuge tubes at 2000 rpm for 30 minutes at 20ºC.

6. After centrifugation, three separate layers comprising of RBC at the bottom, Ficoll-Paque and plasma at the top are obtained. The PBMC are seen as an interphase between the Ficoll-Paque and plasma layer.

7. Carefully remove the plasma layer without disturbing the cells above the Ficoll-Paque. Aspirate the cells from all three centrifuge tubes and place them in a new centrifuge tube.

8. Centrifuge the aspirate at 2000rpm for 10 minutes.

9. Discard the supernatant. Add 5ml of PBS. Mix well and centrifuge at 2000rpm for 10 minutes.

10. Discard the supernatant. Add 1.5ml of PBS, mix well and centrifuge again at 2000rpm for 10 minutes.

11. Discard the supernatant. The washed cells are mixed in 400µl of PBS.

12. The cells are stored as two aliquots of 200µl mixed with 200µl of RNA Later at -80ºC.

Preparation of phosphate buffered saline

Materials required:

NaCl	8gm
KCl	0.2gm
Na_2HPO_4	1.15gm
KH_2PO_4	0.2gm
Distilled water	1000ml

Procedure

1. Dissolve the salts in distilled water and make up the volume to 1000ml.

2. Adjust the pH to 7.4 with 1N NaOH.

3. Autoclave at 121ºC for 15 min at 15lb.

4. The autoclaved solution is stored at 4ºC. For every use PBS is diluted with sterile MilliQ water to a dilution that is one in tenth of the original concentration. The pH is checked and adjusted before every use.

Designing of primers and probes for CMV mRNA RT-PCR

The target RNA selected for reverse transcriptase PCR was the Immediate Early-1 mRNA. The gene sequence for the same was retrieved from the nucleotide blast on Pubmed.org. The primers were designed using the free online software Primer 3. The primers were ordered from Sigma-Aldrich, Bangalore, India.

Reconstitution of primers and probes

Two sets of primers and probes were ordered for the standardization of mRNA quantitative real time reverse transcriptase PCR. The primers and probes were reconstituted with Tris EDTA buffer. The quantity of buffer to be added for the reconstitution was given in the manufacturer's instruction. After reconstitution the final concentration of the primers and probes was 100pmol/µl. The reconstitution of the primers was carried out in a biosafety cabinet after wiping the surfaces with alcohol and RNase Away solution.

The final quantity of the aliquots and the number of aliquots of the primers and probes were calculated. Appropriate numbers of 0.6ml microtubes were labeled with the details of the primers and probes. The tubes were then placed under UV light for 30 minutes inside a

biosafety cabinet. The primers and probes were then aliquoted into these microtubes. The microtubes were then placed in a cryobox and stored at -20ºC until further use.

CMV DNA PCR:

The CMV DNA was extracted from whole blood aliquots and real time quantitative PCR was performed on the Rotor-Gene 3000 thermal cycler (Corbett Research, Sydney, Australia). The gene targeted for the DNA PCR is the immediate early gene. The primers designed for the PCR target a 150 base pair region of this gene. The lower limit of detection of the assay was already determined to be 100 copies/ml and the assay was standardized in the Department of Clinical Virology for routine testing.

DNA extraction protocol

Materials required

Qiamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) containing:

QIAamp Mini Spin Columns

Collection Tubes (2 ml)

Buffer AL

Buffer AW1

Buffer AW2

Buffer AE

Proteinase K

1.7 ml micro centrifuge tube

Handcare gloves, Mask

Plastic apron

Biosafety cabinet

Procedure:

1. Add 20µl of Proteinase K to 200µl of the sample in a 1.5ml of micro centrifuge tube.

2. Add 200µl of Buffer AL to the sample. Mix well by pulse-vortexing for 15 seconds.

3. Incubate the above mixture at 56ºC for 10 minutes in a dry bath.

4. Centrifuge the sample for a brief time to remove the moisture on the lids.

5. Add 200µl of 100% ethanol to the sample and mix well by pulse-vortexing for 15 seconds.

6. Centrifuge the sample to remove the moisture from the inside of the lids.

7. Transfer the above mixture to a Qiagen Mini spin column which is placed on a 2ml collection tube.

8. Centrifuge the Mini spin column at 8000rpm for one minute.

9. The flow through along with the collection tube is discarded and the mini spin column is placed on a fresh collection tube.

10. Add 500µl of buffer AW1 to the mini spin column and centrifuge the sample at 8000rpm for one minute.

11. Discard the collection tube with the flow through. Transfer the mini spin column onto a fresh collection tube.

12. Add 500µl of AW2 buffer to the spin column and centrifuge this at 14,000 rpm for 3 minutes.

13. Discard the collection tube with the flow through. Transfer the mini spin column onto a fresh collection tube and centrifuge at 14,000 rpm for 1 minute at 4ºC.

14. Discard the collection tube and place the mini spin column in a 1.5ml micro centrifuge tube. Add 200µl of elution buffer AE to the spin column and incubate at room temperature for one minute.

15. Centrifuge the above at 8000rpm for one minute.

CMV DNA Real time PCR

Materials required:

Qiagen Norox mastermix buffer (Qiagen, Hilden, Germany)

Nuclease free water

Forward primers

Reverse primers

Taqman probes

Cooling block

Pipette

Pipette tips

Handcare gloves

Biosafety cabinet

Dirty room

Procedure:

The concentrations of the different components of the PCR mix for one reaction are given below:

Reagents	**Quantity(µl)**
Qiagen Norox mastermix buffer	12.5
Forward primer	0.075
Reverse primer	0.075
Taqman probes	0.05
Nuclease free water	2.7
DNA extract	10
Total volume	**25**

1. The mastermix is prepared for the appropriate number of reactions with the above template. The mastermix is prepared in the clean room.

2. Appropriate number of 0.5ml PCR tubes were labeled appropriately.

3. 15µl of the freshly prepared mastermix was aliquoted to the PCR tubes. The PCR tubes are kept on the cooling block.

4. The DNA extract is added to the appropriately labeled PCR tubes in the dirty room. The extracts are added in a biosafety cabinet without light or air currents as the probes are light sensitive.

5. The tubes are loaded into the Rotor-Gene 3000 thermal cycler (Corbett Research, Sydney, Australia) which is kept in another room.

6. The cycling conditions for the reactions are as follows:

CYCLE	**TEMPERATURE(ºC)**	**TIME**
HOLD	95	15 minutes
CYCLING (50 cycles)	95	45 seconds
	60	75 seconds

The total time required for the completion of the run is 160 minutes.

The quantitation of the DNA was performed using in house standards prepared by cloning.

The internal control used for the extraction procedure and the PCR conditions was the amplification of the GAPDH gene. The cycling conditions for the amplification of the GAPDH gene were similar to that for the amplification of the CMV DNA PCR.

CMV mRNA RT-qPCR:

The 72 kDa splice variant of the IE-mRNA was the target for the RNA PCR. The RNA was extracted from the PBMC stored at -80ºC.

RNA extraction protocol

Materials required:

Qiagen Rneasy minikit (Qiagen, Hilden, Germany) containing:

- RNeasy Mini Spin Columns
- Collection Tubes (1.5 ml)
- Collection Tubes (2 ml)
- Buffer RLT
- Buffer RW1
- Buffer RPE
- RNase-Free Water

70% Distilled water

Pipette (100 - 1000µl)

Pipette (20 - 200µl)

Pipette tips

Hand care gloves, Surgical mask

Plastic apron

Centrifuge

Vortex

Biosafety cabinet

Procedure:

The Qiagen RNeasy minikit was used for extraction of RNA from the PBMC samples. The method as recommended by the manufacturers was followed for the extraction process.

1. The PBMC sample is thawed at 4ºCelsius.

2. 600µl of RLT buffer is added to the microtube containing the PBMC sample. This is vortexed and centrifuged.

3. One volume of 70% ethanol is added to the microtube. The sample is mixed by pipetting and centrifuging should be avoided in this procedure.

4. Transfer 700µl of the sample to an RNeasy minispin column and centrifuge at 9500rpm for 15 seconds. The flow through is discarded and the 2ml collection tube is reused. The remaining sample is transferred to the spin column and centrifuged at 9500 rpm for 15 seconds.

5. 500µl of RPE buffer is added to the spin column and the spin column is centrifuged at 9,500 rpm for 15 seconds. The flow through is discarded and the collection tube is reused.

6. 500µl of RPE buffer is again added to the spin column and centrifuged at 9,500 rpm for 2 minutes. The flow through and collection tube are discarded.

7. The spin column is transferred to a new 2ml collection tube and centrifuged at 13,500 rpm for 1 minute.

8. The spin column is transferred to the 1.5ml microtube. 50µl of RNase free water is added to the spin column. This is centrifuged at 9,500 rpm for 1 minute. This step elutes the RNA.

DNase treatment of RNA

Materials required:

DNAse enzyme with buffer

EDTA

0.5 ml microtubes

Procedure:

1. 1µl of DNase enzyme is mixed with 1µl of the buffer in a 0.5ml microtube tube. This step is carried out in the clean room.

2. 8µl of the RNA extract is added to the above mixture.

3. The mixture is incubated at 37ºC for 30 minutes in a thermal cycler.

4. The DNase enzyme is inactivated by adding 1µl of EDTA and incubating at 65ºC for 10 minutes in a thermal cycler.

5. The DNAse treated RNA can be used for reverse transcriptase PCR. This pretreatment step ensures that the DNA present in the sample does not give a false positive result with reverse transcriptase PCR.

Reverse transcriptase PCR

Materials required

Qiagen One step RT PCR Kit (Qiagen, Hilden, Germany)

Nuclease free water

Forward and reverse primers, probes

Cooling block

Pipette, pipette tips

Procedure

The concentrations of the different components of the PCR mix for one reaction are given below:

Reagents	Quantity (µl)
One step RT-PCR mastermix	12.5
Reverse transcriptase enzyme	0.25
Forward primer	0.15
Reverse primer	0.15
Probe	0.1
Nuclease free water	1.85
DNase treated RNA extract	10
Total volume	**25**

1. The mastermix is prepared for the appropriate number of reactions with the above template. The mastermix is prepared in the clean room.

2. Appropriate number of 0.5ml PCR tubes are labeled.

3. 15µl of the freshly prepared mastermix is aliquoted to the PCR tubes. The PCR tubes are kept on the cooling block.

4. The RNA extract is added to the appropriately labeled PCR tubes in the dirty room.

5. The tubes are loaded into the Rotor-Gene 3000 thermal cycler (Corbett Research, Sydney, Australia).

6. The cycling conditions for the reactions are as follows:

CYCLE	**TEMPERATURE(ºC)**	**TIME**
HOLD 1 (cDNA conversion)	45	15 minutes
HOLD 2	95	10 minutes
CYCLING X 45 CYCLES	95	25 seconds
	53	30 seconds
	72	60 seconds

The total reaction takes approximately 180 minutes to complete.

The quantitation of the mRNA was performed using inhouse standards produced by cloning.

The internal quality control used for the PCR is the Endogenous retrovirus (ERV3) RNA. ERV3 standards were used for quantitation of the cells present in the sample based on the principle that every human cell has two copies of ERV3.

Determination of the limit of detection of the CMV IE-mRNA RT PCR assay

The lower limit of detection of the assay was determined by replicate testing of serial dilutions of the known standards used for quantitation. The lowest dilution standard (S5) for the assay contained 30,000 copies/ml. The standards were serially diluted so as to obtain 1/10, 1/100 and 1/1000 dilutions of S5. The copy numbers in the corresponding dilutions were 3000 copies/ml, 300 copies/ml and 30 copies/ml. The dilutions were then tested in triplicates on three separate days. The minimum concentration of the copies detected was then calculated and an average of the copies was taken to determine the lower limit of detection of the assay.

ERV3 RT-qPCR

The RNA extract used for the CMV mRNA estimation was used for the quantitation of ERV3. The real time quantification of ERV3 RNA was done using the two step reverse transcriptase method. After DNase treatment the extract was used for cDNA conversion. The cDNA was then used for real time quantification.

cDNA conversion of ERV3 RNA

The following template was used for cDNA conversion of ERV3:

Reagent	Amount(µl)
5x buffer	4
dNTP	0.8
Random primer	2
0.1 M DTT	2
RNase Out	0.5
MMLV Reverse Transcriptase enzyme	0.2
MilliQ water	0.5
DNase treated RNA	10
Total volume	**20**

1. The mastermix is prepared for the appropriate number of reactions with the above template. The mastermix is prepared in the clean room.

2. Appropriate number of 0.5ml PCR tubes is labeled.

3. 15µl of the freshly prepared mastermix is aliquoted to the PCR tubes. The PCR tubes are kept on the cooling block.

4. The RNA extract is added to the appropriately labeled PCR tubes in the dirty room. The extracts were added to the PCR tubes in a biosafety cabinet with the light and air current switched off.

5. The PCR tubes are then loaded on the ABI Veriti thermal cycler (Applied Biosystems, Foster City, CA, USA), which is kept in another room.

The temperature settings for the cDNA conversion are as follows:

Cycle	Temperature(ºC)	Time(minutes)
Hold (cDNA conversion)	37	60
Stop (Termination of enzyme activity)	95	5

The resulting cDNA was subjected to real time PCR. The template used for the PCR is as follows:

Reagents	Quantity(µl)
Qiagen Norox mastermix buffer	12.5
Forward primer	0.075
Reverse primer	0.075
Taqman probes	0.05
Nuclease free water	2.7
cDNA extract	10
Total volume	**25**

1. The mastermix was prepared for the appropriate number of reactions with the above template. The mastermix is prepared in the clean room.

2. Appropriate number of 0.5ml PCR tubes are labeled.

3. 15µl of the freshly prepared mastermix was aliquoted to the PCR tubes. The PCR tubes are kept on the cooling block.

4. The DNA extract is added to the appropriately labeled PCR tubes.

5. The tubes are loaded into the Rotor-Gene 3000 thermal cycler.

The cycling conditions for the PCR are given below:

Cycle	**Temperature(°C)**	**Time**
Hold 1	45	15 minutes
Hold 2	95	10 minutes
Cycling (50 cycles)	95	25 seconds
	56	30 seconds
	72	60 seconds

The reaction takes approximately 150 minutes to complete.

The quantitation of ERV3 was done using in-house standards for ERV3.

RESULTS

RESULTS

Sample collection:

The study was initiated in July, 2012 after appropriate clearance was obtained from the Institutional Review Board of the college. A total of 162 patients were enrolled into the study from July, 2012 to September, 2013. Informed consent was taken from all patients before enrolling into the study. 132 patients were followed up completely for a period of 100 days in the post-transplant period. A total of 2467 samples were collected from all the study patients and all the samples were stored as four aliquots. The patient compliance regarding the sample collection is depicted in the following graph:

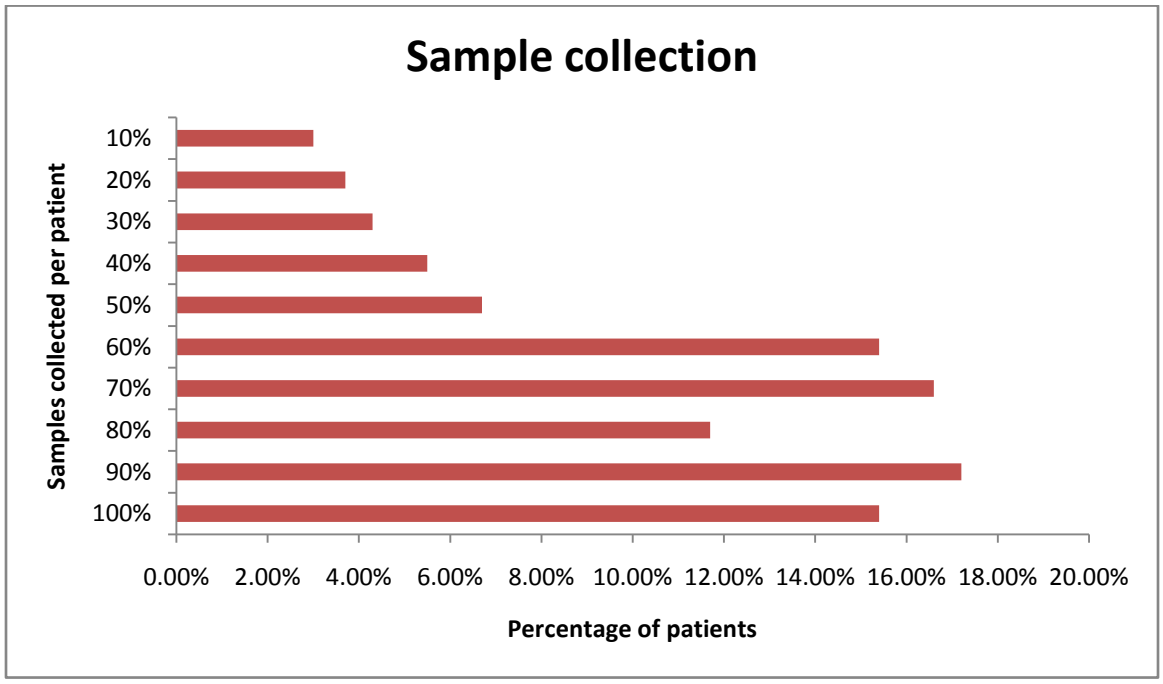


Figure 1: Graph depicting the patient compliance in sample collection.

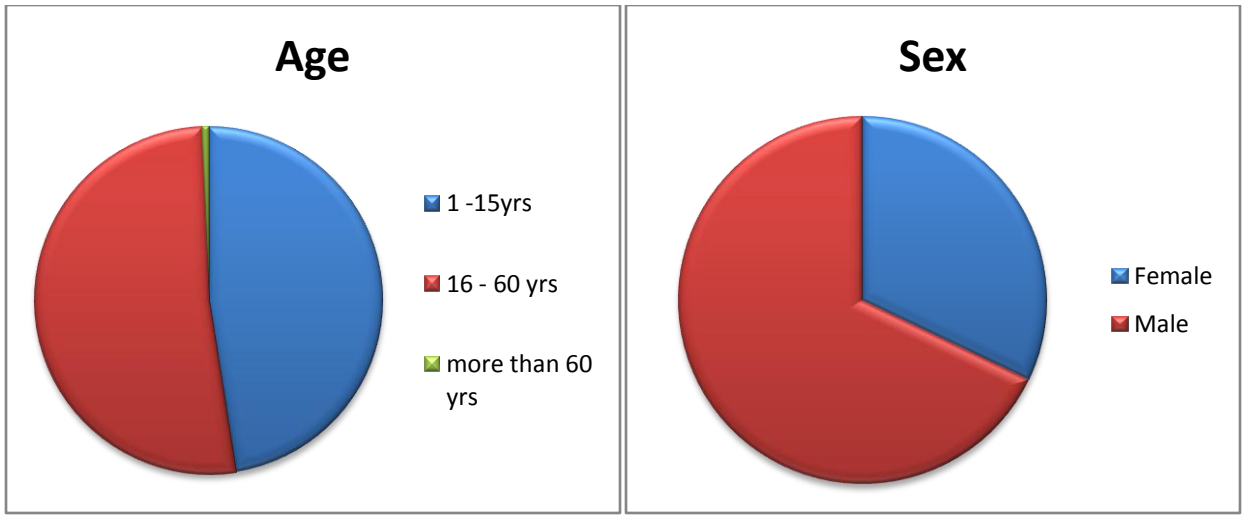
All 21 samples were collected from 25 patients, who form 15.4% of the study group. Only 38 patients gave less than 10 samples for the study and most of them were recruited in the later part of the study where follow up for 100 days was not possible.

The samples were transported at 4ºC to the Department of Clinical Virology in containers with ice packs. The samples were processed on the same day of collection. In the laboratory, each patient was allotted a study number (eg: CS1) and four 1.7ml micro centrifuge tubes were labeled with the study number as well as the day of collection of that sample. Two aliquots of 1ml and 200µl of whole blood were stored in the micro centrifuge tubes at -20ºC. The rest of the sample was used to separate PBMCs using the Ficoll-Paque based density gradient separation technique. After the PBMCs were separated, it was stored in RNA LATER solution at -80ºC as two aliquots.

Demographic data for 162 patients:

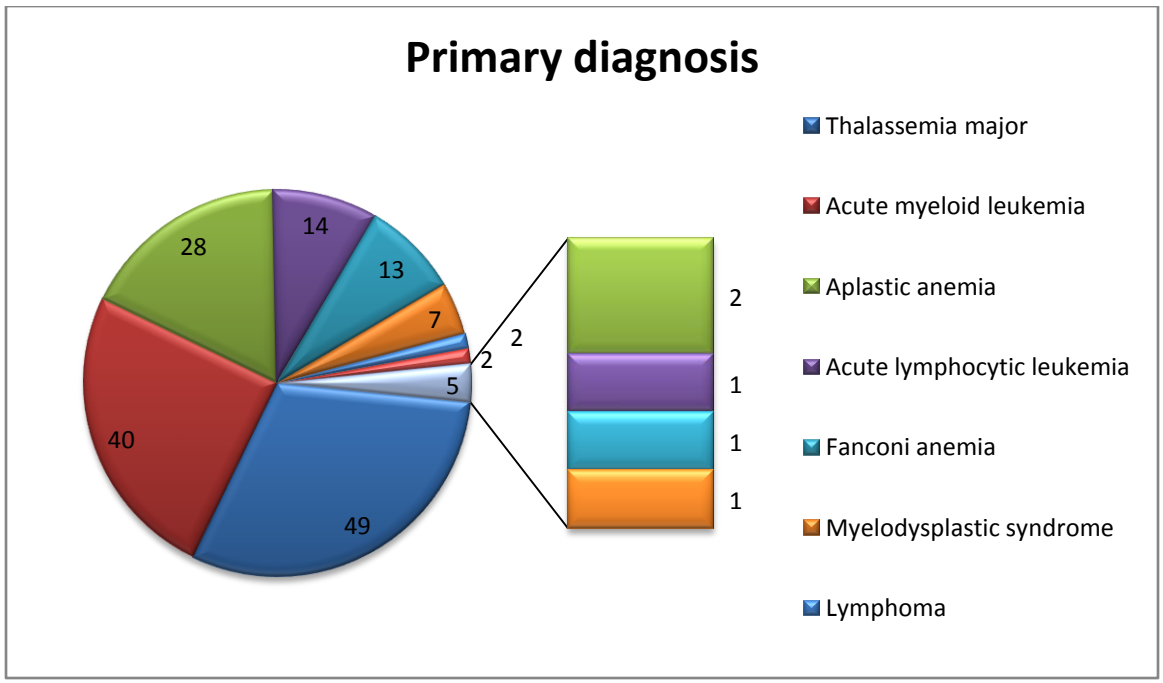
Age: The age groups of the patients ranged from 1 year to 63 years. 77 patients (47.5%) of the patients belonged to the pediatric age group with ages ranging from 1 to 15 years.

Sex: Out of 162 patients, 52 were female patients and 110 were male patients.



Primary diagnosis:

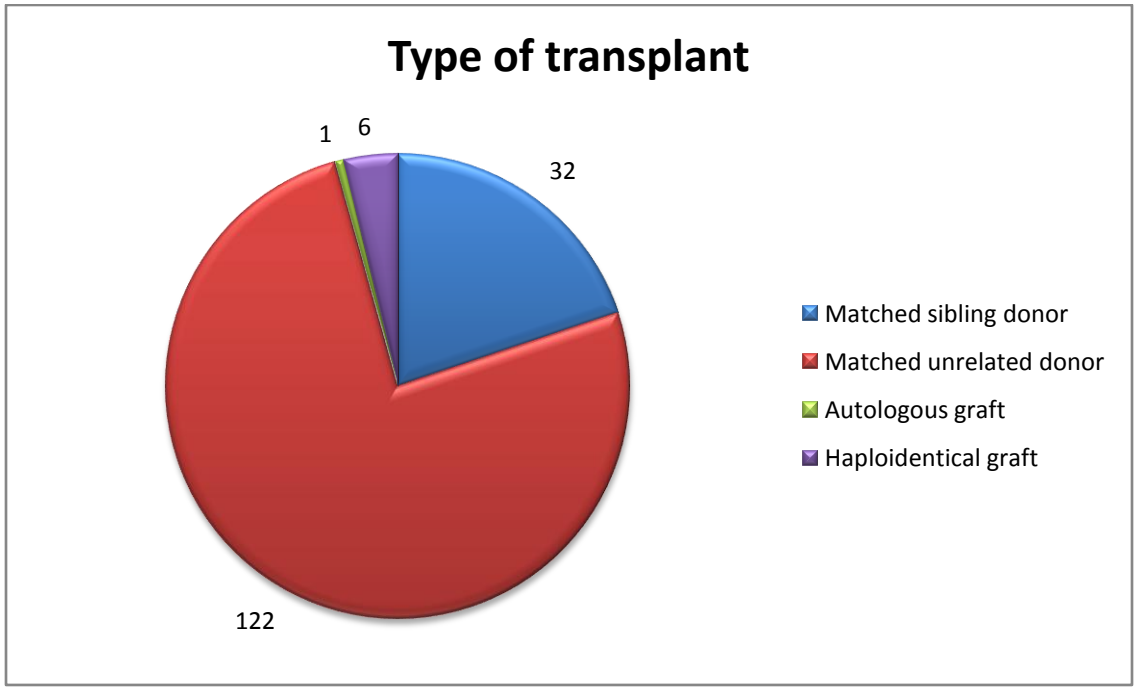
The patients enrolled into the study underwent HSCT for malignant and non-malignant causes.



The major non-malignant condition for which HSCT was performed was for thalassemia major while the major malignant condition was acute myeloid leukemia.

Type of transplant:

The source of stem cells for the graft can be a sibling, an unrelated donor (allogeneic) or the patient itself (autologous). All allogeneic sources undergo HLA typing and matching with the recipient. In the event of no matched donors, a haploidentical graft can be taken from a donor.

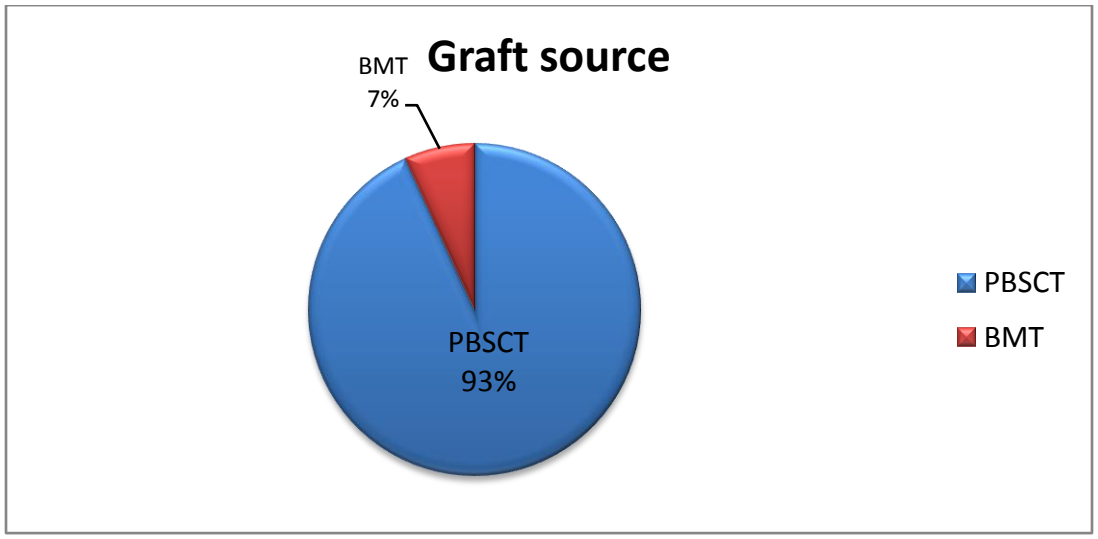


Matched unrelated donors formed 75.3% of the donor group. The other major group of donors was the matched sibling donors who formed 19.7% of the group.

Graft source:

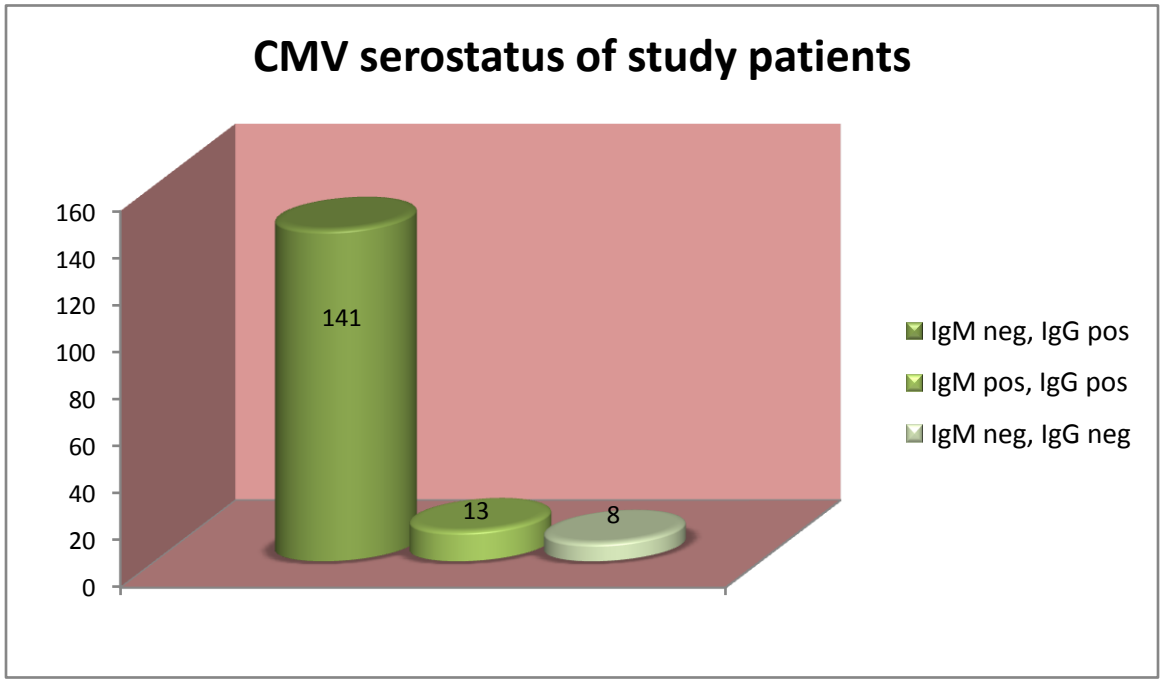
Stem cells can be harvested from the peripheral blood (PBSCT) or the bone marrow (BMT).

The source of the graft for the patients in the study group is represented in the graph below.



CMV Serostatus:

The CMV serostatus of all patients was determined by measuring their CMV IgG antibodies just before transplantation.



The CMV serostatus of the patients reflects the epidemiology of the virus in the Indian subcontinent. Only 4.9% of the study population was not exposed to the virus; the majority (95%) of the study group had been exposed to the virus before the transplantation.

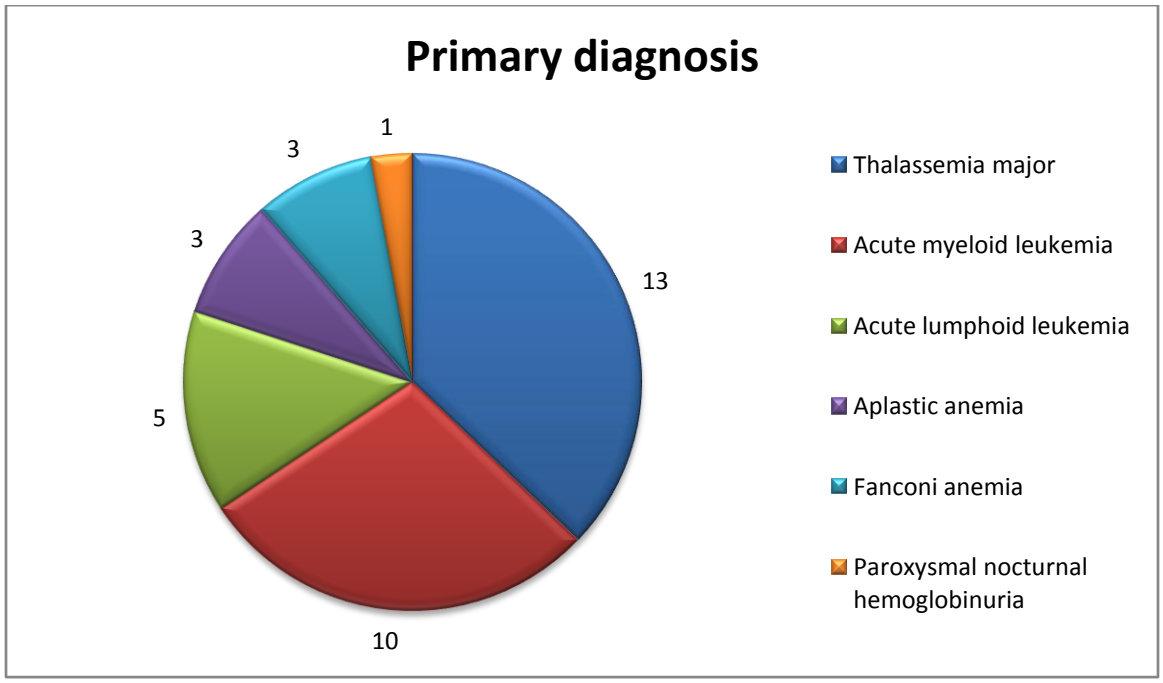
CMV DNA testing:

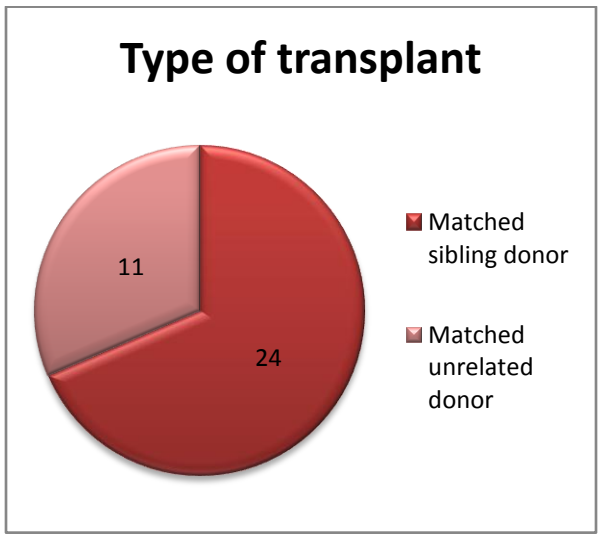
Reactivation of CMV in the post-transplant period was determined by CMV DNA levels monitored by real time quantitative PCR. Routine testing for CMV DNA is performed on the samples of these patients if there is clinical suspicion of the disease or as routine twice or thrice in the post-transplant period. As part of the study, the weekly samples from all patients were stored at -20ºC. The patients who reactivated for CMV had CMV DNA levels more than 1000copies/ml. These patients were then selected and their samples were retrieved from storage and CMV DNA PCR was performed on them later.

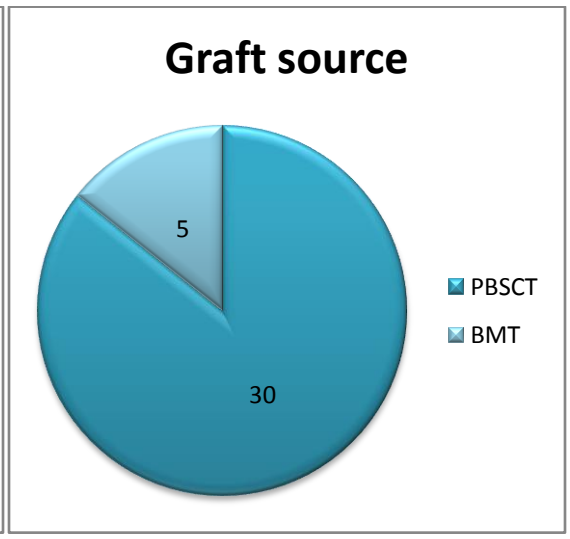
Rate of reactivation:

Thirty five of the 162 patients (21.6%) reactivated during the course of the study. The age groups ranged from 2 to 50 years. 16 patients (45.7%) were in the pediatric age group, between 2 and 15 years. The reactivators comprised 11 female patients and 24 male patients.

The primary diagnosis of the reactivators, the type of donor and the graft source is depicted in the charts below:

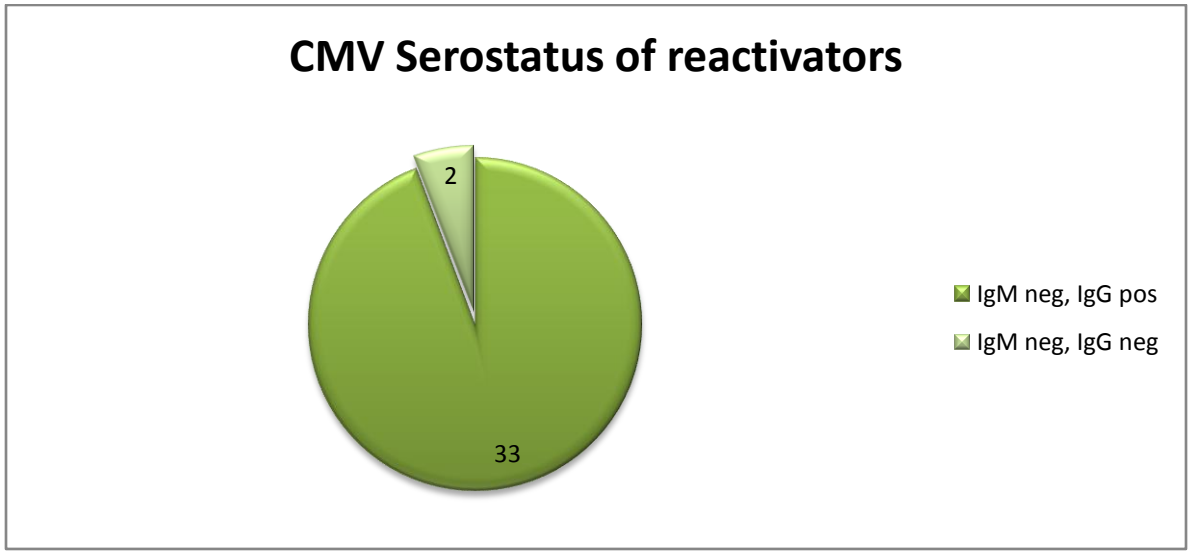






CMV Serostatus of reactivators:

Of the 35 patients, 33 tested negative for CMV IgM and positive for CMV IgG, indicating past exposure to HCMV and a possible latent status of the virus in these patients. Only two patients tested negative for both CMV IgM and CMV IgG indicating that the patients did not have an exposure to the virus before transplantation.



CMV DNA Serial monitoring:

25 patients, who had a complete set of samples, were selected from the reactivators and serial DNA monitoring was undertaken for these patients. The samples of these patients were taken out of the freezer and thawed and CMV DNA PCR was conducted on these samples.

Comparison of presenting CMV DNA values and peak CMV DNA values:

Serial CMV DNA values of all the patients were measured and graphs were plotted for each individual patient.

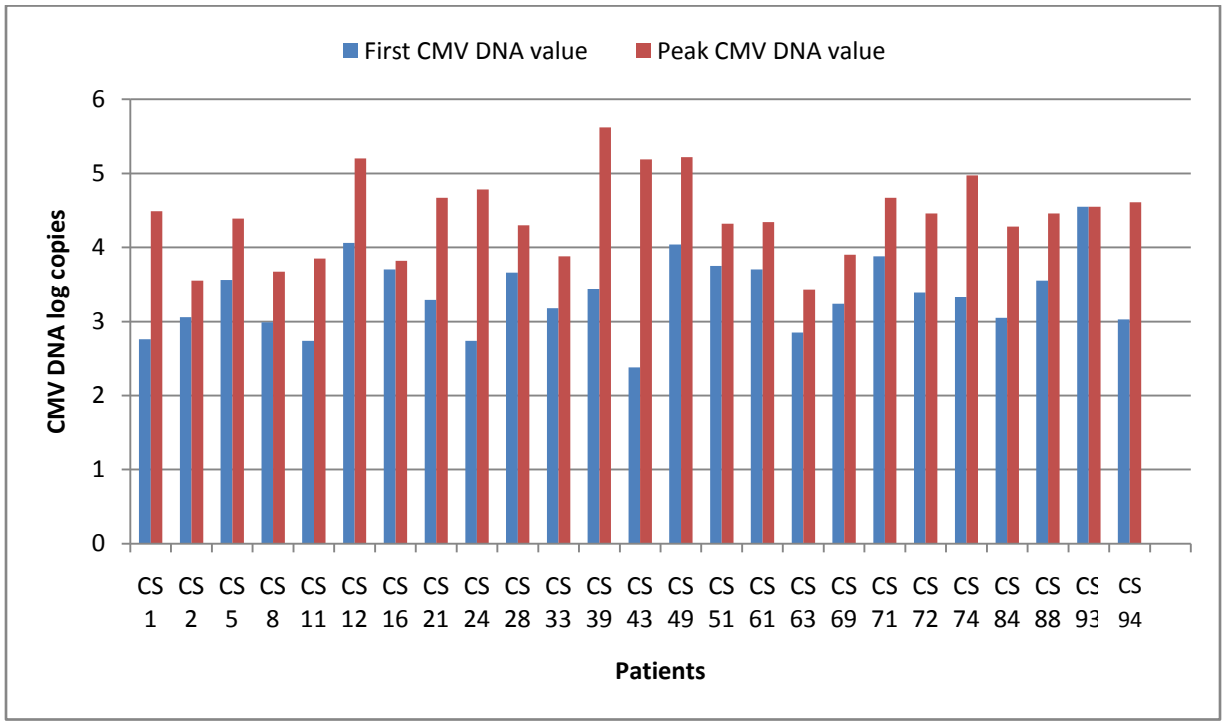


Figure 2: Graph comparing the CMV DNA values at presentation and peak CMV DNA values of reactivators.

Out of 25 patients who were serially monitored for CMV DNA, 24 patients had CMV DNA values that were low at the time of presentation with higher values of DNA appearing later during the reactivation. One patient had the highest DNA values at the time of presentation.

Day of reactivation:

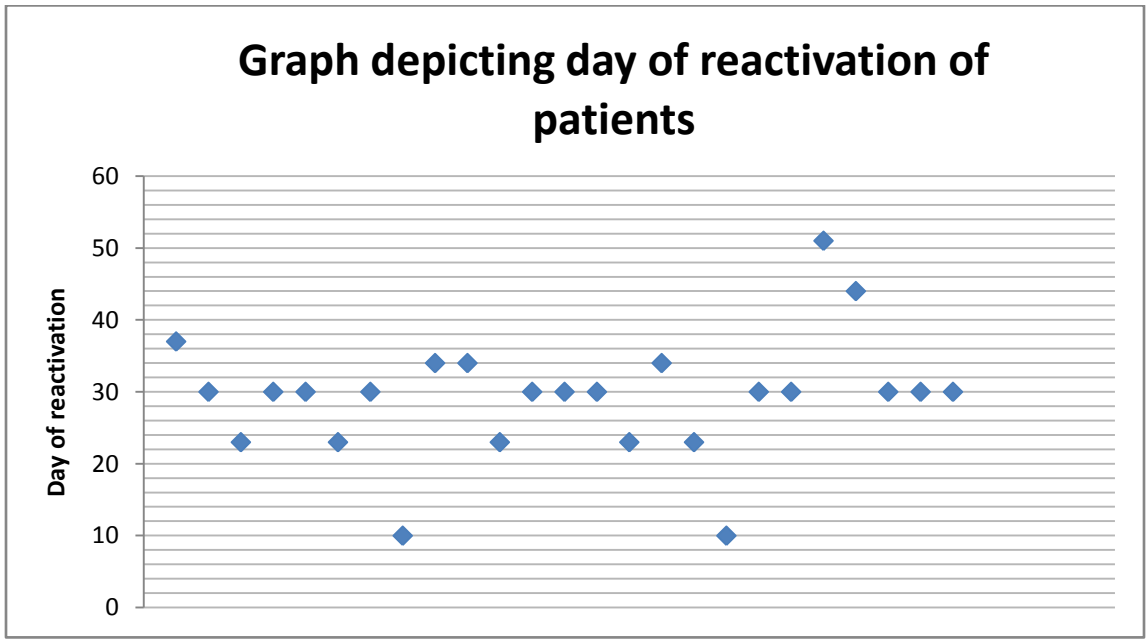


Figure 3: Graph depicting the day of CMV reactivation of patients in the post-transplant period

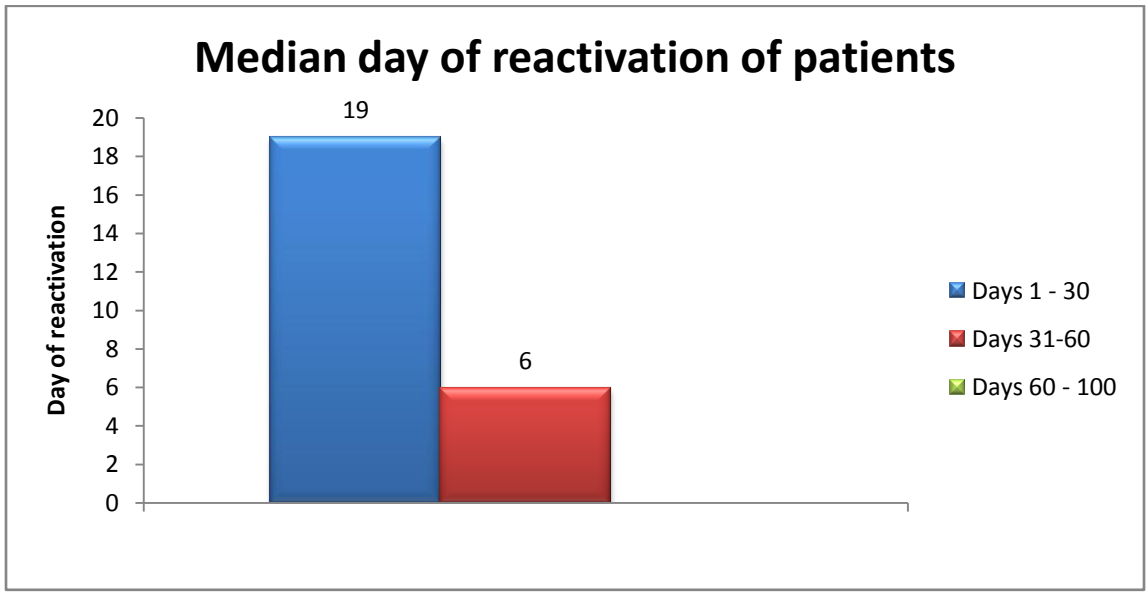


Figure 4: Graph depicting the number of patients showing reactivation at various time intervals

Twelve of 25 patients (48%) of the study group reactivated on Day 30 in the post-transplant period while5 (20%) of them reactivated on Day 23. 2 patients (8%) reactivated in the pre engraftment period at Day 10. 23 patients (92%) reactivated in the engraftment period which has been established to be the time period in the post-transplant period when patients are most susceptible to CMV reactivation with the virus. This has been attributed to the poor cell mediated immunity seen in patients during the period.

Comparison of peak CMV DNA levels in reactivators:

The peak CMV DNA values of all the patients are represented in the following graph.

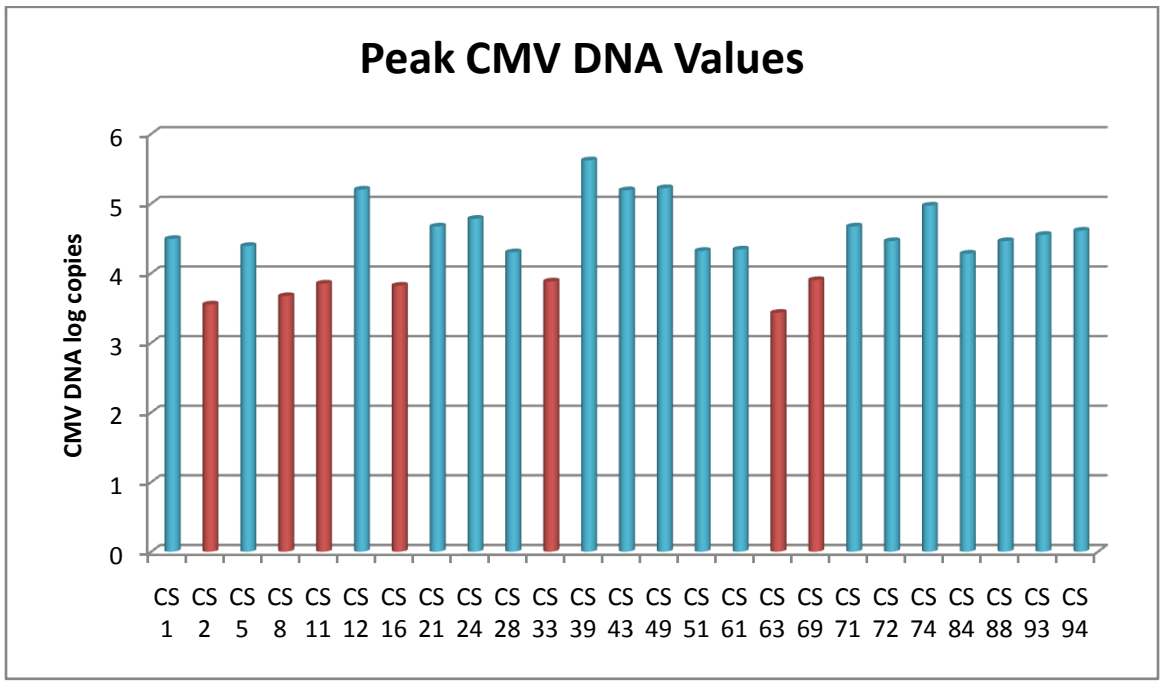


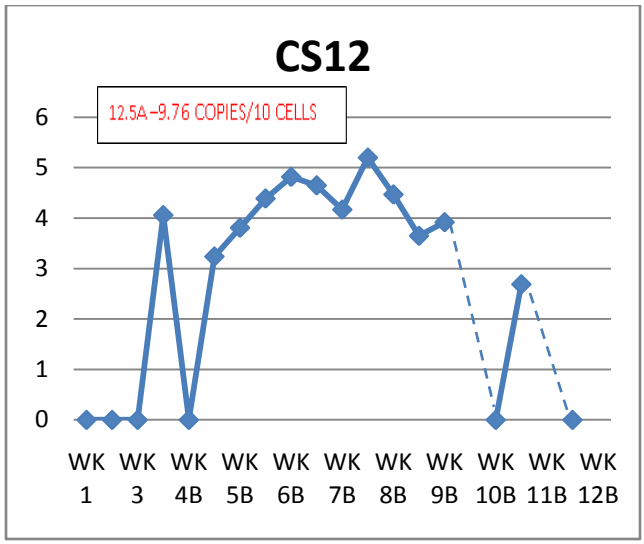
Figure 5: Graph depicting the peak CMV DNA values of all reactivators.

Of 25 patients, 7(28%) consistently showed CMV DNA values below 10,000 copies/ml while 18 (72%) of the patients had CMV DNA values above 10,000 copies/ml.

CMV IE-mRNA measurement:

Of 25 patients, CMV IE-mRNA was monitored during periods of reactivation for 15 patients. 7 patients showed detectable mRNA levels during reactivation. The mRNA levels were quantitated and the copy numbers are represented as copies per number of cells. The cell numbers were determined based on ERV-3 levels in the stored PBMC samples.

The time points at which mRNA tested positive for these patients are represented in the graphs below. The X axis represents the points in time when the samples were collected, Y axis represents the CMV DNA values in log copy numbers and the RNA values are represented in boxes on the graph.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12.5A –9.76 COPIES/10 CELLS
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5
4
3
2
1
0
WK 1
WK 3
WK 4B
WK 5B
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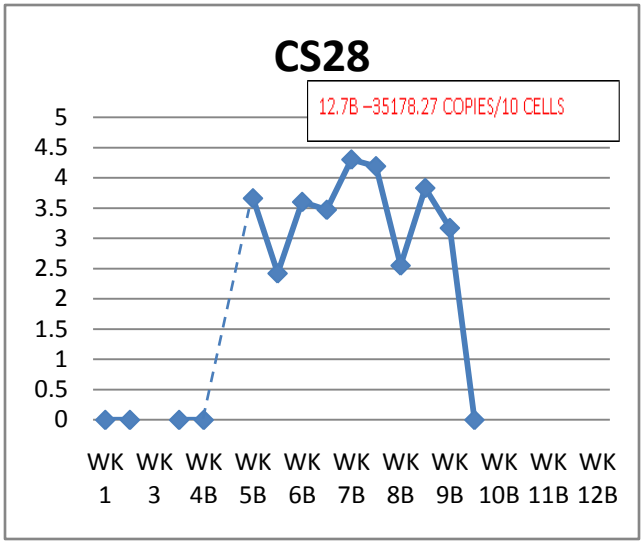
WK 7B
WK 8B
WK 9B
WK 10B
WK 11B
WK 12B


CS28
12.7B –35178.27 COPIES/10 CELLS
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4.5
4
3.5
3
2.5
2
1.5
1
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0
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WK 3
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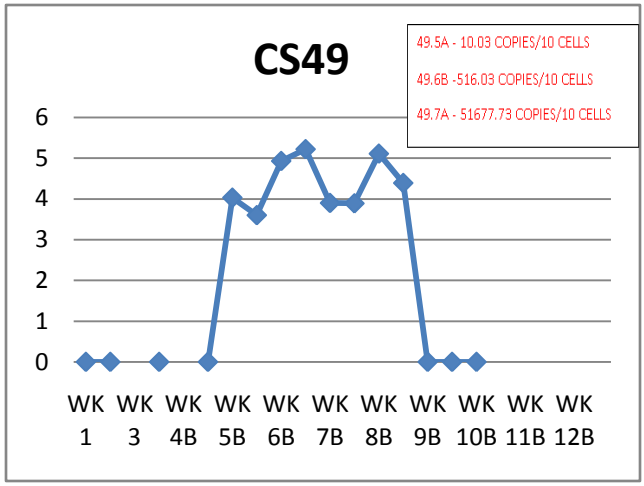
WK 5B
WK 6B
WK 7B
WK 8B
WK 9B
WK 10B
WK 11B
WK 12B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49.6B -516.03 COPIES/10 CELLS
49.7A - 51677.73 COPIES/10 CELLS
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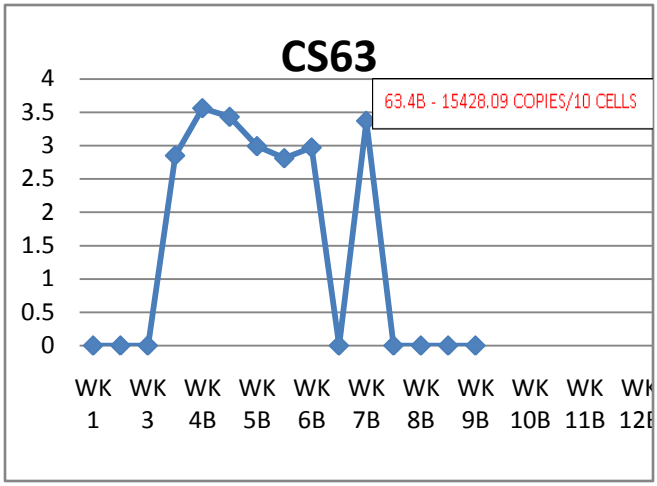
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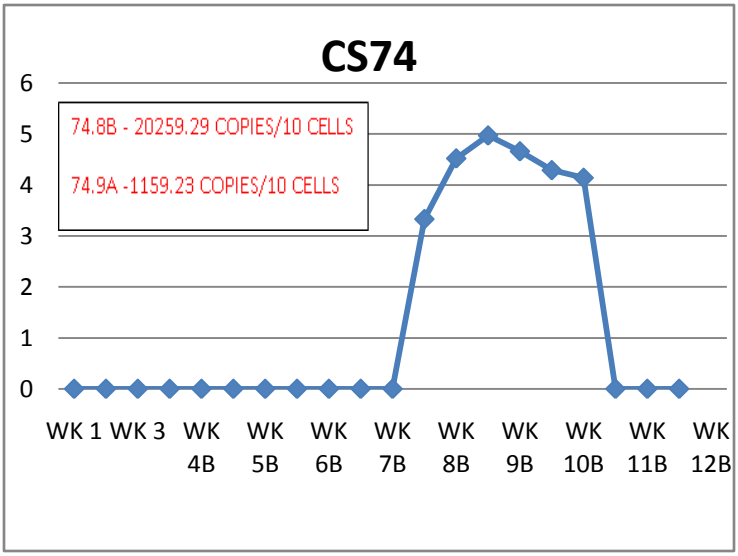
WK 10B
WK 11B


CS74
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74.9A -1159.23 COPIES/10 CELLS
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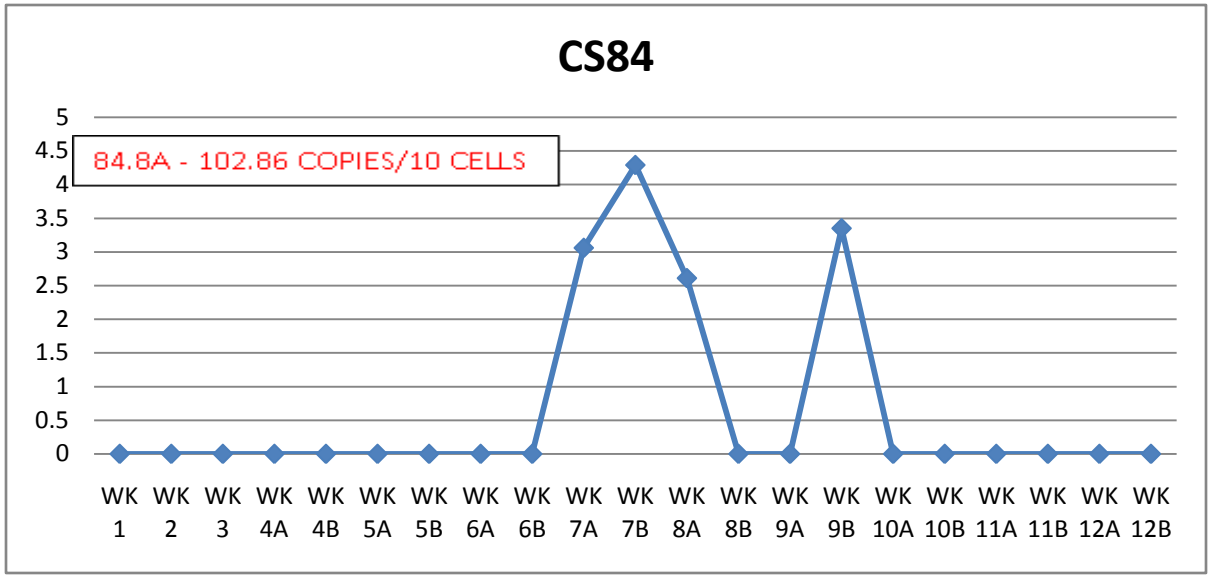
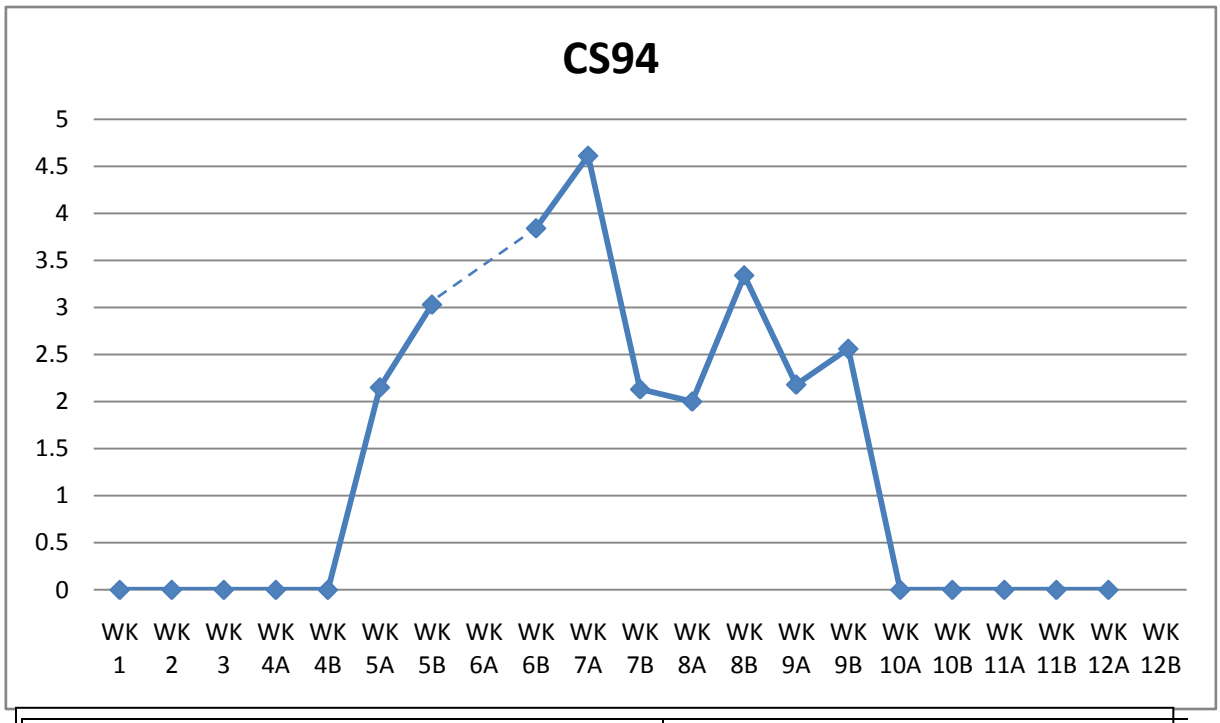
WK 10B
WK 11B
WK 12B





CS94.4B	104.48 COPIES/10 CELLS
CS94.6B	1719.46 COPIES/10 CELLS
CS94.7A	324.52 COPIES/10 CELLS
CS94.9A	376.37 COPIES/10 CELLS

CMV IE-mRNA detection:

Seven patients (46.6%) of the 15 patients tested for IE-mRNA showed detectable mRNA levels.

Characteristics of 7 patients who had detectable IE-mRNA:

Pt no:	Age	Sex	Diagnosis	CMV Sero-status (IgG)	Day of first positive DNA	Day of first positive mRNA	Peak CMV DNA values (copies/ml)	Peak CMV mRNA values (copies/10 cells)
CS12	M	47	AML	+ve	23	30	1,57,555	9.76
CS28	M	5	FA	+ve	34	47	19,763	35,178.27
CS49	F	47	AML	+ve	30	30	1,67,499	51,677.73
CS63	F	27	ALL	+ve	23	27	2,666	15,428.09
CS74	M	2	TM	+ve	51	54	93,871	20,259.29
CS84	M	36	AA	+ve	44	51	19,298	102.86
CS94	M	18	TM	+ve	30	27	40,829	1,719.46

Table 1: Table depicting the characteristic features of patients with detectable CMV IE-mRNA levels. AML – Acute Myeloid Leukemia; FA – Fanconi Anemia; ALL – Acute Lymphoid Leukemia; AA – Aplastic Anemia; TM – Thalassemia major

IE-mRNA was detectable in all patients after CMV DNA was detected except for one patient in whom CMV IE-mRNA was detected 3 days before DNA levels were detected. All the patients showed detectable mRNA levels while their CMV DNA levels were increasing in the initial phase of reactivation. Only one patient had detectable CMV IE-mRNA while the CMV DNA level was declining. However this patient also showed an increase in the CMV DNA level one week later

Peak CMV DNA values for RNA positive samples:

Study number	DNA level (copies/ml)
CS12	1,57,555
CS28	19,763
CS49	1,67,499
CS63	2,666
CS74	93,871
CS84	19,298
CS94	40,829

Mean HCMV DNA values for these patients: 71,640.14

Peak CMV DNA values for RNA negative values:

Study number	DNA level (copies/ml)
CS1	22,230
CS2	3,527
CS5	16,456
CS8	4,721
CS11	6,853
CS24	60,045
CS33	7,561
CS39	4,81,482

Mean CMV DNA values for these patients: 75,359.37

There was no significant difference between the mean peak CMV DNA values of the patients who tested positive for CMV IE-mRNA and the patients who tested negative for CMV IE-mRNA.

Limit of detection of CMV IE-mRNA RT-qPCR:

The copies detected by the RT-qPCR for the IE-mRNA are depicted below.

Copy numbers (copies/ml)	**Day 1**	**Day 2**	**Day 3**
3000	3053	773	5879
3000	4885	1153	1234
3000	2598	3448	3065
300	Not detected	1662	963
300	1849	Not detected	Not detected
300	1227	290	Not detected
30	Not detected	Not detected	Not detected
30	Not detected	Not detected	Not detected
30	Not detected	Not detected	Not detected

The limit of detection of the assay was calculated by calculating the mean of the values detected in the lowest dilution in the three separate occasions. The lower limit of detection of the assay was determined to be 1198 copies/ml.

DISCUSSION

DISCUSSION

Hematopoietic stem cell transplantation has evolved to be a life changing mode of therapy for malignancies and non-malignant conditions like thalassemia major and aplastic anemia. CMV is a major viral opportunistic pathogen in bone marrow transplant patients in the post-transplant period. Monitoring for CMV reactivation by CMV DNA PCR in the post transplant period is an essential investigation performed in the post transplant period. However no consensus is available on the CMV DNA levels at which preemptive therapy should be initiated for such patients. Moreover the drug of choice for preemptive therapy is now ganciclovir which in itself is a bone marrow toxic drug (144). The other major concern with ganciclovir therapy is the emerging trends of resistance of CMV to this drug, leaving the physicians with limited options for therapy (145).

We evaluated the efficiency of the use of CMV IE-mRNA as a new marker of CMV reactivation in bone marrow transplant patients in the post transplant period. Earlier studies conducted on the use of CMV IE-mRNA as a marker met with problems such as false positivity with CMV DNA. This problem was alleviated by subjecting the RNA extracts with DNase enzyme before performing the reverse transcriptase PCR. Patients who underwent HSCT in our hospital were enrolled into our study. CMV reactivation in these patients was monitored by measuring their CMV DNA levels in whole blood using real- time PCR. CMV IE-mRNA levels were looked for in reactivators using real-time reverse transcriptase PCR.

A total of 162 patients were enrolled into the study. Among them 77 patients (47.5%) belonged to the pediatric age group and 110 of them were male patients. The major malignant condition for which transplantation was done was acute myeloid leukemia and the major non malignant condition was thalassemia major. The donor group comprised mainly of matched unrelated donors and peripheral blood stem cells were the major graft source.

The epidemiology of CMV infection varies with geographical regions and studies from India show that the viral infection is highly endemic in the subcontinent. The CMV serostatus of the patients before transplantation correlates with national statistics with 95% showing elevated CMV IgG values, indicating past exposure to the virus. Only eight patients (5%) tested negative for both CMV IgM and IgG.

According to global statistics, 60–70% of patients undergoing bone marrow transplantation with high risk factors undergo CMV reactivation in the post-transplant period. However, in our study, only 35 of 162 (21.6 %) of the study population had CMV reactivation during the study period. Among the 35 reactivators, 33 patients were CMV seropositive even before transplantation. 2 patients were CMV seronegative before transplantation.

Indian data regarding the rate of reactivation of CMV in HSCT patients is limited and one study conducted by Finny G J *et al* in 2001 records a reactivation rate of 42.8% of 31 patients studied. The data from our study reveals a low rate of reactivation in this group of patients as compared to Western data and it may be attributed to the presence of

neutralizing antibodies (anti gB/AD-1) in patients who did not develop the disease (151). In another study conducted by Finny *et al* in Indian renal transplant patients, similar findings were noted. The study revealed a higher incidence of neutralizing anti gB/AD-1 antibodies along with high avidity CMV anti-IgG antibodies in patients who did not develop CMV reactivation in the post transplant period as opposed to the patients who reactivated (152).

Another possibility to be considered is the prevalent genotypes in the Indian population. Many studies have been conducted in developed countries on the prevalent genotypes of CMV and the impact they have on the outcome of patients in the post-transplant period (153,154). However, extrapolation of such data to the Indian population may not be accurate as the seroprevalence to the virus is high in India compared to developed nations. Genotype association studies have been conducted in the Indian subcontinent on perinatal and congenitally acquired CMV infection in neonates (155,156). The study conducted by Gandhoke *et al* on symptomatic infants in Delhi showed that the prevalent genotypes found in these infants were gB 1, 2 and 3. Genotype gB 3 was the more frequently isolated genotype from symptomatic infants (156). However no such data is available on HSCT patients from the Indian subcontinent.

Among the reactivators, 92% reactivated in the engraftment period established to be the post-transplant period when patients are most susceptible to CMV reactivation . This has been attributed to poor cell mediated immunity seen in patients during this period. Only two patients showed increased viral loads in the pre-engraftment period.

The CMV DNA level which indicates a “true” CMV infection has not been determined; the cutoff level at which preemptive therapy is initiated in our hospital currently is 1000 copies/ml. Of 25 patients, 7(28%) consistently showed CMV DNA values below 10,000 copies/ml while 18 (72%) had CMV DNA values above 10,000 copies/ml. Several studies have established cutoffs from 1,000 to 10,000 copies/ml of CMV DNA for initiation of preemptive therapy. The values depend on a number of factors and can vary depending on the seroprevalence of CMV in the respective geographical regions and the assay used to determine the CMV DNA levels (11). However there remains the need to establish an accurate cutoff for initiation of preemptive therapy.

The CMV IE-mRNA PCR was preliminarily performed for 15 patients and 7(46.6%) patients showed detectable mRNA levels during reactivation. IE-mRNA was detectable in all patients after CMV DNA was detected except for one patient in whom CMV IE-mRNA was detected 3 days before DNA levels were detected. The limit of detection of the assay was determined to be 1198 copies/ml. The low sensitivity of the assay may be attributed to this limit of detection of the assay. The lower limit of detection of mRNA should be improved so as to detect lower copy numbers of the mRNA. This could also be one of the reasons why the mRNA was detected only after the CMV DNA was detected.

Another reason for the low sensitivity of the assay may be attributed to the alternate splicing mechanisms of the Immediate Early-1 region of the HCMV genome. The splicing mechanisms at this region produce four different transcripts which are selectively produced at varying concentrations during the different steps of viral

replication (27). Also the major spliced region of the mRNA that codes for the 72-kDa protein, which was the region of the genome that was targeted by our IE-mRNA assay, can further undergo varied splicing depending on the replication stage of the virus (26). The numerous splicing mechanisms present at this part of the viral genome may be responsible for the low sensitivity of this assay.

All the patients showed detectable mRNA levels while their CMV DNA levels were increasing in the initial phase of reactivation. Only one patient had detectable CMV IE-mRNA while the CMV DNA level was declining. However this patient also showed an increase in the CMV DNA level one week later. This finding reflects the fact that CMV IE- mRNA detection represents a true reactivation and if untreated can lead to CMV disease.

The role of IE-mRNA as a potential marker of CMV reactivation cannot be ruled out. The assay developed in the study for detection of mRNA should be standardized further to detect lower copy numbers and the other splice variants should be considered to improve the sensitivity of the assay.

LIMITATIONS OF THE STUDY

1. The quality of RNA after extraction from PBMCs was not checked with UV spectrophotometric analysis.
2. The lower limit of detection of the assay was assessed to be 1198 copies/ml and needs to be improved to be able to detect lower copies of the mRNA.
3. Alternate splicing mechanisms of mRNA need to be addressed while designing primers and probes. Also the alternate splice variants should be checked for in patients who tested negative for the 72 kDa variant of this mRNA.

CONCLUSIONS

CONCLUSIONS

The rate of reactivation of CMV in HSCT patients in our study was found to be 21.6%. This was surprisingly low compared to the Western data which records reactivation rates of 60-70%.

Among 25 patients who were continuously monitored for CMV DNA levels, 7(28%) showed values which were consistently below 10,000 copies/ml.

CMV IE-mRNA is an indicator of the reactivation of HCMV in bone marrow transplant patients in the post-transplant period. However the sensitivity of the IE-mRNA RT-qPCR needs to be improved so as to detect lower copies of the mRNA.

Alternate splicing mechanisms of the IE-mRNA should be carefully evaluated to design primers and probes that ensure detection of all the splice variants by RT-qPCR. The alternate splice variants also need to be tested in the patients who were negative for the 72kDa variant of the IE-mRNA.

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ANNEXURE

CMV STUDY CASE RECORD

S. No: …… Date: ……..

Name: Hosp No:

Age: yrs Sex: **M** **F** Diagnosis:

Type of SCT: HSD/ HFD / MUD Date of SCT: ……/……/…….

Type of conditioning: Graft source: PBSC / BM

Date of Neutrophil engraftment (ANC > 0.5 x 10^9/L): ……../……/……..

Date of Platelet engraftment (Plt > 20 x 10^9/L): ……/……/…….

Acute GVHD: Y / N If Yes: Date on onset: ……/……/…….

Overall Grade of GVHD: 0-1 / II / III / IV

CMV viremia: Y / N If Yes, Date on onset: ……/……/…….

CMV copies at diagnosis: …… copies

Randomization: Y / N; Date of randomization: ……/……/……..

Group randomized into: **I / II**

Anti-viral therapy started: Ganciclovir / Foscarnet / Cidofovir

Date of starting treatment: ……/……/……. Date of stopping treatment: ……/……/…….

Outcome of Rx: Complete resolution/ Partial resolution/ Developed CMV disease/ Died

CMV disease: Y/N; Ste: Lungs/GIT / CNS / BM / Liver; Date of Dx: ……/……/……

Relapse of CMV: Y / N; Date of relapse: ……/……/…….

Present status: Alive / Death If dead, cause – GVHD / infection / CMV / Relapse

Department of Haematology & Department of Clinical Virology

Christian Medical College,

Vellore, 632 004

Assent Form

Title of Study:

A study of the kinetics of CMV viremia in patients undergoing bone marrow transplantation and identification of cutoffs for initiation of ganciclovir therapy in patients who show reactivation of CMV infection

Principal investigators:

Dr Rayaz Ahmed, Assistant Professor, Clinical Haematology

Dr. Sangeeta Susan Thomas, PG Registrar, Microbiology

Dr. Biju George, Professor Clinical Haematology

Dr. Asha Mary Abraham, Professor Clinical Virology

Why are we doing this study?

You have been asked to participate in a research study. One of the major complications that can occur after bone marrow transplant is a viral infection called as Cytomegalovirus. This infection can involve the lungs and/or the liver and intestines and is difficult to treat. Normally we try to detect this virus in the blood and start treatment before it involves the organs of the body. However, the medicines used to treat this infection are costly and causes a number of side effects most importantly a decrease in all the blood counts leading to infection and bleeding.

Normally we start treatment at very low levels of virus. This may not be required and it may be safe to start treatment only when the levels rise. This study is aimed at finding out if it is reasonable to start treatment only after the levels have increased. This means that in a number of people, treatment with this costly medicine can be avoided and the side effects can also be avoided.

What will happen during the study?

You will be monitored after transplant with blood samples to look for evidence of viral infection. This is done routinely for all patients after transplant. You will be included in the study only if the tests for CMV come positive. If you consent for the study, you will either be started on a medicine called ganciclovir once the levels rise beyond 1000 copies or your levels will be monitored and medicine started only once the levels have increased beyond a specified level. You will be monitored serially with blood samples to look for improvement in the levels.

Are there good things and bad things about the study?

If you are randomized into the arm where the levels are monitored, it is possible that you may not require treatment at all.

This will help in reducing the cost and side effects associated with this treatment.

There is a worry whether by waiting there is a possibility of the viral infection affecting various organs. You will be monitored very closely while on follow up and treatment can be started at any time.

Who will know about what I did in the study?

If you are a part of this study your name and address will not be given to anyone without your consent unless required by law. If you would like, results of this study will be made available to you when it is finished.

Can I decide if I want to be in the study?

You have every right to decide whether you want to participate in this study. Nobody will be angry or upset if you do not want to be in the study. We are talking to your parents/ legal guardians about the study and you should talk to them about it too. If you do not take part in this study, you will be treated with our standard practice that is to start the medicine as soon as the levels go beyond 1000 copies/ml.

Assent:

I was present when…….. read this form and gave his/her verbal assent.

_____

Signature/ Thumb impression Date

_____ _____ _____

Name of person who obtained consent Signature Date

INFORMED CONSENT TO PARTICIPATE IN A RESEARCH STUDY

Investigator: Dr. Biju George

Study Title: A study of the kinetics of CMV viremia in patients undergoing bone marrow transplantation and identification of cutoffs for initiation of ganciclovir therapy in patients who show reactivation of CMV infection.

Protocol Number:

Subject /Parent Initials:	Subject Number:	Subject Birth Date:
_____	_____	_____

INTRODUCTION

You are being asked to take part in a research study. This research study gathers information about a new type of treatment. Dr. Biju George is the primary person conducting the study.

You will take part in the study only if you want to. It is important that you have the information about this study that you need in order to decide if you want to participate. Dr. Biju George will talk to you about the study, and answer your questions. Please take your time making your decision.

This study has been approved by an ethics committee.The ethics committee helps protect subjects in research studies.

This study involves starting treatment with a medicine for a viral infection called cytomegalovirus (CMV) at different time points when the test becomes positive. This study will attempt to answer whether this modification will reduce the amount of people who require treatment for this infection and others in future may benefit from the information learned in this study.

Approximately 70 subjects will take part in this study. Your participation in this study will last for approximately 3 months after your transplant, the side effects are acceptable, and the investigator believes it is in your best interest.

PURPOSE OF THE STUDY

You are being asked to take part in this study because approximately 50% of patients who have an allogeneic transplant will develop a viral infection called CMV. We check for this infection routinely in all patients using a blood test called as PCR. If the test is positive, you are routinely started on a drug called as ganciclovir to reduce the infection. This medicine however has a number of side effects importantly a rapid reduction in blood counts. In some patients, this infection may improve by itself if the immune system of the body controls it. Hence in many hospitals, doctors may wait to see if the levels rise before starting this treatment. The purpose of this study is to see whether starting this treatment later will help in reducing the number of persons who will require this treatment.

TEST ARTICLE

Nil

STUDY PROCEDURES

You will be asked to sign this informed consent form before any further treatment is given.

If the PCR test for CMV becomes positive, you need to receive a drug called as ganciclovir. You will receive this drug either as soon as the test becomes positive or when the levels increase. You will be monitored regularly after starting treatment to look for recovery from the infection. The medicine will be stopped after 14 days of treatment once the PCR is negative. You will be followed up regularly after stopping the medicine to look for any symptoms of this disease coming back again.

ACTIVE STUDY PERIOD

The study covers a period of 3 months following transplant. You may ask if you have any clarification.

RISKS

Infection with CMV generally involves the blood but in 10% of patients it can affect the lungs, liver or the stomach. The medicine is aimed at reducing the chance of involvement of these organs in the body. We do not think that by starting the medicines later, there will be a higher risk. The medicines on the other hand can cause a marked reduction in blood counts in more than 50 -60% of patients. This can leading to infections and bleeding.

BENEFITS ASSOCIATED WITH THE STUDY

The number of people who will need treatment for CMV infection will be lower and this will lead to a lesser number of side effects. Others may benefit from the information learned in this study.

ALTERNATIVES TO PARTICIPATION

We will continue to treat as soon as the PCR becomes positive which is our standard practice.

CONFIDENTIALITY

Your records from the study are confidential unless law requires certain people to see them. Your name or identifying information will not be used in reports or publications resulting from this study.

PAYMENT OR REIMBURSEMENT

Nil

PAYMENT FOR TREATMENT OF RESEARCH-RELATED INJURY

Nil

COSTS

You will not have any additional costs as a participant in this study.

LIMITS OF FINANCIAL COMPENSATION

Nil

CONTACTS

If you have questions about your rights as a research subject, please call Dr. L. Jeyaseelan, Member Secretary, Ethics Committee or Dr. Nihal Thomas, Deputy Chairperson, Research & Ethics Committees at +91-416-2284294

If you have questions about this study or if you think you have had an injury related to your participation in this study and you want information on treatment, please call Dr. Biju George +91-416-2282169

VOLUNTARY PARTICIPATION

Your participation in this study is voluntary. You do not have to agree to participate. If you do not take part in this study, you treatment will continue as is our standard practice.

CONSENT

Information describing this research study has been explained to me. I have read and understood this consent form. All my questions have been answered to my satisfaction. I voluntarily consent for my son/daughter to participate in this research study and to the release of my medical records as described in this form.

I do not give up my legal rights by signing this consent form. I will receive a signed and dated copy of this consent form.

Study Title: A study of the kinetics of CMV viremia in patients undergoing bone marrow transplantation and identification of cutoffs for initiation of ganciclovir therapy in patients who show reactivation of CMV

Study Number:

Name of Study Doctor: _____

Patient Number/Initials: _____/_____

Date of Birth / Age: _____

Patient Name: _____

Parent/Legal guardian's Name: _____

		Please initial Box (Subject)
1	I , _____, have read and understood the preceding information describing this research study and my questions have been answered to my satisfaction. I voluntarily consent to my son/daughter to participate in this research study and to the release of my medical records as set forth under the “Confidentiality” section.	( )
2	I have received /will receive a copy of the signed Patient Information/ Informed Consent.	( )
3	I understand that my son’s/daughter’s participation is voluntary and that he/she is free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	( )
4	I understand that the investigators, the Ethics Committee and regulatory authorities will not need my permission to look at my son’s/daughter’s health records in respect of the current study and any further research that may be conducted in relation to it, even if he/she withdraws from the trial. I agree to this access. However, I understand that my son’s/daughter’s identity will not be revealed in any information released to third parties or published.	( )
5	I agree to the transfer of my son’s/daughter’s personal anonymized data to other countries in accordance with local laws and guidelines	( )

6 I agree not to restrict the use of any data or results that arise from this study provided such a use is only for scientific purpose(s) ( )

7 I agree for my son/daughter to take part in the above study. ( )

_____	_____		_____
Name of Patient	Signature of the parent /legal guardian*	Thumb impression	Date of Signature

_____	_____	_____
Name of Study Doctor	Signature	Date of Signature

(* If a patient has limited ability to read and write, an impartial witness should be present during the entire informed consent discussion and his/her legally acceptable representative should sign on patient's behalf). In these instances the patient places his/ her left thumb impression in the place of the signature.

Patient's Legally Acceptable Representative's Statement: ☐ **NA**

I, as the patient's legally acceptable representative, was present during the consenting

procedure and understand the preceding information describing this study. All of the questions regarding the study and the patient's participation in it have been answered to my satisfaction and that of the patient. I state that all aspects of the study were clearly presented during the consent procedure. The patient is willing to participate in the study and I sign below on his/her behalf testifying to this effect.

Name of the Patient: _____

Name of the Legally Acceptable Representative: _____

Relationship to the Patient: _____

Signature of the Legally Acceptable Representative:_____

Date and signature:_____

Witness Declaration of Patient's Informed Consent ☐ **NA**

By signing the consent form I attest that the information was accurately explained to and apparently understood by the patient and the legally acceptable representative (if applicable) and that informed consent was freely given by the patient.

(* A witness is not required unless the subject is unable to read (e.g. blind or illetrate) or unless indicated in the protocol. If a witness is present, the witness must observe the entire informed consent process).

Date _____ Signature _____

(Impartial Witness)

Name of Witness: _____

Address of Witness: _____

_____

Comments:

_____

_____

_____

INSTITUTIONAL REVIEW BOARD (IRB)
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Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

July 6, 2012

Dr. Rayaz Ahmed
Assistant Professor
Department of Hematology
Christian Medical College
Vellore 632 004

Sub: **Fluid Research grant project NEW PROPOSAL:**
A study of the kinetics of CMV viremia in patients undergoing bone marrow transplantation and identification of cutoffs for initiation of ganciclovir therapy in patients who show reactivation of CMV infection
Dr. Rayaz Ahmed, Assistant Professor, Hematology, Dr. Sangeeta Susan Thomas, Microbiology, Dr. Asha Mary Abraham, Clinical Virology, Dr. Biju George , Dr. Alok Srivastava, Dr. Vikram Mathews, Dr. Aby Abraham, Dr. Auro Vishwabandhya, Haematology, Dr. O C Abraham, Medicine, Mr. Santosh, Clinical Virology, Mr. Prasanna, Biostatistics

Ref: IRB Min. No. 7841 dated 25.04.2012

Dear Dr. Ahmed,

The Institutional Review Board (Silver, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project titled "A study of the kinetics of CMV viremia in patients undergoing bone marrow transplantation and identification of cutoffs for initiation of ganciclovir therapy in patients who show reactivation of CMV infection" on April 25, 2012.

The Committees reviewed the following documents:

1. Format for application to IRB submission
2. CMV study case Record
3. Informed Consent Form and Assent Form (English)
4. Cvs of Drs. Asha Mary Abraham, Biju George, Sangeeta Susan Thomas, Sangeeta Susan Thomas, Alok Srivastava, Auro Viswabandya, Vikram Mathews, Aby Abraham.

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Chairperson, Ethics Committee

Dr. L. Jeyaseelan, MSc, PhD
Secretary, Research Committee, IRB

Dr. Alfred Job Daniel, MS Ortho
Chairperson, Research Committee &
Principal

Dr. Nihal Thomas
MD MNAMS, DNB(Endo), FRACP(Endo), FRCP(Edin)
Deputy Chairperson
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

5. A CD containing documents 1 – 4

The following Institutional Review Board (Ethics Committee) members were present at the meeting held on April 25, 2012 in the CREST/SACN Conference Room, Christian Medical College, Bagayam, Vellore 632002.

Name	Qualification	Designation	Other Affiliations
Dr. George Thomas	MBBS, D Ortho	Chairperson (IRB) & Orthopaedic Surgeon, St. Isabel Hospital, Chennai & Former Editor, Indian Journal of Medical Ethics	External
Dr. DJ Christopher	BSc, MBBS, DTCD, DNB, FCCP	Professor of Pulmonary Medicine, CMC	
Dr. Jayaprakash Muliyil	BSC, MBBS, MD, MPH, DrPH(Epid), DMHC	Academic Officer, CMC	
Mr. Samuel Abraham	MA, PGDBA, PGDPM, M.Phil, BL.	Legal Advisor, CMC.	
Mrs. Mary Johnson	M.Sc	Professor of Maternity Nursing, CMC.	
Dr. Vathsala Sadan	M.Sc, Ph.D	Professor of Community Health Nursing, CMC	
Dr. P. Zachariah	MBBS, PhD	Retired Professor , Vellore	External
Mrs. S. Pattabiraman	BSc, DSSA	Social Worker, Vellore	External
Dr. Nihal Thomas	MD MNAMS DNB(Endo)FRACP (Endo) FRCP(Edin)	Secretary IRB (EC)& Dy. Chairperson (IRB), Professor of Endocrinology & Addl. Vice Principal (Research), CMC.	

We approve the project to be conducted as presented.

The Institutional Review Board expects to be informed about the progress annually of the project, any serious adverse events occurring in the course of the project, any changes in

INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE
VELLORE 632 002, INDIA

Dr. George Thomas, D Orth
Editor, Indian Journal of Medical Ethics
Chairperson, Ethics Committee

Dr. Alfred Job Daniel, MS Ortho
Chairperson, Research Committee &
Principal

Dr. L. Jeyaseelan, MSc, PhD
Secretary, Research Committee, IRB

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Deputy Chairperson
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

the protocol and the patient information/informed consent and requires a copy of the final report.

Yours sincerely,

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

Secretary
Institutional Review Board
(Ethics Committee)
Christian Medical College
Vellore - 632 002, Tamil Nadu, India

Study no:	Age	Sex	Hospital no:	Diagnosis	Pre-trnsplnt c	Dt of trnsplnt	Wk 1	Wk 2
CS1	12	M	132729F	Acute Leukemia	25/7/2012	27/7/2012	1/8/2012	9/8/2012
							NEG	
CS2	12	F	812122D	Thalassemia Major	26/7/2012	1/8/2012	4/8/2012	13/8/2012
							NEG	NEG
CS5	20	M	698750C	Fanconi Anemia with MDS	30/7/2012	4/8/2012	7/8/2012	13/8/2012
							NEG	NEG
CS8	50	M	197848F	AML M4	13/8/2012	17/8/2012	20/8/2012	27/8/2012
							NEG	NEG
CS11	32	F	208315F	AML	20/8/2012	23/8/2012	27/8/2012	3/9/2012
							NEG	NEG
CS12	47	M	146223F	AML	20/8/2012	24/8/2012	27/8/2012	3/9/2012
							NEG	NEG
CS16	13	M	012158F	Thalassemia Major	23/8/2012	29/8/2012	3/9/2012	10/9/2012
							NEG	NEG
CS21	5	M	854928D	Thalassemia Major	10/9/2012	17/9/2012	17/9/2012	24/9/2012
							NEG	1974
CS24	16	F	257096F	AML-M1	17/9/2012	21/9/2012	24/9/2012	1/10/2012
							NEG	NEG
CS28	5	M	929189D	Fanconi Anemia	24/9/2012	30/9/2012	1/10/2012	8/10/2012
							NEG	NEG
CS33	18	F	270704F	AML	8/10/2012	11/10/2012	15/10/2012	22/10/2012
							NEG	NEG
CS39	32	M	325017D	ALL		18/10/2012	25/10/2012	1/11/2012
							NEG	NEG
CS43	15	M	111409F	Thalassemia Major	29/10/2012	2/11/2012	5/11/2012	12/11/2012
							NEG	NEG
CS51	35	F	157226D	ALL	12/11/2012	15/11/2012	19/11/2012	26/11/2012
							NEG	NEG
CS61	50	M	321898F	AML	3/12/2012	7/12/2012	10/12/2012	
							NEG	
CS49	47	F	281462F	AML M1	8/11/2012	14/1/2012	10/1/2013	17/1/2013
							NEG	NEG

CS63	27	F	223319F	ALL	10/12/2012	14/12/2012	17/12/2012	24/12/2012
							NEG	NEG
CS69	1	M	924418D	Thalassemia Major	24/12/2012	31/12/2012	3/1/2013	10/1/2013
								1741
CS71	22	M	178570F	ALL	31/12/2012	5/1/2013	7/1/2013	14/1/2013
							NEG	NEG
CS72	15	M	159795F	Aplastic anemia	3/1/2013	8/1/2013	10/1/2013	17/1/2013
							NEG	NEG
CS74	2	M	714847D	Thalassemia Major	7/1/2013	14/1/2013	17/1/2013	24/1/2013
							NEG	NEG
CS84	36	M	370602F	Aplastic anemia		8/2/2013	11/2/2013	18/2/2013
							NEG	NEG
CS94	18	M	575868C	Thalassemia Major	25/2/2013	1/3/2013	4/3/2013	11/3/2013
							NEG	NEG
CS88	6	F	345821F	ALL	14/2/2013		21/2/2013	28/2/2013
							NEG	NEG
CS93	11	M	217647F	Thalassemia major	21/2/2013		28/2/2013	7/3/2013
							NEG	NEG

Wk 3		Wk 4		Wk 5			Wk 6		Wk 7			Wk 8	
13/8/2012				30/8/2012			3/9/2012	6/9/2012	10/9/2012			17/9/2012	20/9/2012
NEG				NEG			570	NEG	NEG			22230	73636
	30/8/2012			3/9/2012	6/9/2012	10/9/2012	13/9/2012		17/9/2012	20/9/2012	24/9/2012		27/9/2012
	NEG			1152	3527	3220	3496				NEG		NEG
20/8/2012	29/8/2012	30/8/2012		3/9/2012	7/9/2012	10/9/2012	13/9/2012		17/9/2012	20/9/2012	24/9/2012		27/9/2012
NEG	3764	21563		10123	24503	11497	16456		NEG	NEG	NEG		NEG
3/9/2012	6/9/2012			13/9/2012		20/9/2012	24/9/2012		27/9/2012	1/10/2012	4/10/2012		8/10/2012
NEG	NEG			977		2850			4721	2704	NEG		NEG
8/9/2012		17/9/2012		20/9/2012		24/9/2012	27/9/2012		1/10/2012	4/10/2012	8/10/2012		11/10/2012
NEG		NEG		546		6853	1268		3861	6620			NEG
10/9/2012	17/9/2012	20/9/2012		24/9/2012		27/9/2012	1/10/2012		4/10/2012	8/10/2012	11/10/2012		15/10/2012
NEG	11492	NEG		1749		6453	24665		66343	44279	14962		157555
17/9/2012	24/9/2012	27/9/2012		1/10/2012		4/10/2012	8/10/2012		11/10/2012	15/10/2012	18/10/2012		22/10/2012
NEG	NEG	NEG		5006		NEG	2438		6648	NEG	NEG		NEG
1/10/2012		11/10/2012		15/10/2012			18/10/2012		22/10/2012	25/10/2012	29/10/2012		1/11/2012
NEG		1241		5948			13994		44254	46707	11846		23477
8/10/2012	13/10/2012	18/10/2012		22/10/2012		25/10/2012	29/10/2012		1/11/2012	5/11/2012	8/11/2012		15/11/2012
NEG	NEG	NEG		NEG		559	300		6561	60045	1084		4290
15/10/2012	18/10/2012	22/10/2012				29/10/2012	1/11/2012		5/11/2012	8/11/2012	12/11/2012		15/11/2012
	NEG	NEG				4592	264		4046	2978	19763		15625
29/10/2012	5/11/2012	8/11/2012		12/11/2012		15/11/2012	19/11/2012		22/11/2012	26/11/2012	29/11/2012		3/12/2012
NEG	1515	445		4454		7561	345		NEG	NEG	NEG		NEG
10/11/2012	13/11/2012	15/11/2012		19/11/2012		22/11/2012	26/11/2012		29/11/2012	3/12/2012	6/12/2012		10/12/2012
NEG	NEG	NEG		2767		7049	481482		16543	31790	68584		46251
22/11/2012	29/11/2012			6/12/2012		10/12/2012	13/12/2012		17/12/2012	20/12/2012	24/12/2012		27/12/2012
NEG	NEG			240		31524			37679	156348	97305		35002
	10/12/2012	13/12/2012		17/12/2012		20/12/2012			27/12/2012	31/12/2012	3/1/2012		7/1/2013
NEG	5589	8608		NEG		NEG			3125	20800	NEG		NEG
17/12/2012	24/12/2012			31/12/2012		3/1/2013	7/1/2013		10/1/2013	14/1/2013			21/1/2013
NEG	NEG			NEG		5011	819		NEG	21848			12622
24/1/2013	31/1/2013			7/2/2013		11/2/2013	14/2/2013		18/2/2013	21/2/2013	25/2/2013		28/2/2013
	NEG			10922		4014	85633		4212	167499	8054		7752

Wk 8	
20/9/2012	
73636	
27/9/2012	
NEG	
27/9/2012	
NEG	
8/10/2012	
NEG	
15/10/2012	
NEG	
18/10/2012	
29371	
25/10/2012	
NEG	
5/11/2012	
23477	
15/11/2012	
4290	
19/11/2012	
357	
6/12/2012	
NEG	
13/12/2012	
18741	
31/12/2012	
99585	
10/1/2013	
NEG	
24/1/2013	
2416	
4/3/2013	
130147	

31/12/2012	10/1/2013	14/1/2013	17/1/2013	21/1/2013	24/1/2013	28/1/2013	31/1/2013	4/2/2013	7/2/2013	
NEG	703	2272	2666	984	642	946	NEG	2369	NEG	
17/1/2013	24/1/2013	28/1/2013		4/2/2013	7/2/2013	11/2/2013	14/2/2013	18/2/2013	21/2/2013	25/2/2013
8101		4988		NEG	NEG	NEG	NEG	NEG	NEG	NEG
21/1/2013	28/1/2013	31/1/2013	4/2/2013	7/2/2013	11/2/2013	14/2/2013	18/2/2013	21/2/2013	25/2/2013	28/2/2013
	NEG	NEG	7735		46902	7577	7909	21084	14201	588
24/1/2013	31/1/2013	4/2/2013	7/2/2013	11/2/2013	14/2/2013	18/2/2013	21/2/2013	25/2/2013		
	NEG	NEG	2488	8561	29096	409	1985	3524		
31/1/2013	7/2/2013	11/2/2013	14/2/2013	18/2/2013	21/2/2013	25/2/2013	28/2/2013	4/3/2013	7/3/2013	11/3/203
NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	2161	33208
25/2/2013	28/2/2013	4/3/2013	7/3/2013	11/3/2013		18/3/2013	21/3/2013	25/3/2013	28/3/2013	1/4/2013
NEG	NEG	NEG	NEG	NEG		NEG	1142	19298	409	NEG
18/3/2013	21/3/2013	25/3/2013	28/3/2013	1/4/2013	4/4/2013	8/4/2013	11/4/2013	15/4/2013	18/4/2013	22/4/2013
NEG	NEG	NEG	142	1086	NEG	7061	40829	137	101	2229
7/3/2013	11/3/2013	14/3/2013	18/3/2013	21/3/2013	25/3/2013	28/3/2013	1/4/2013	4/4/2013	8/4/2013	11/4/2013
NEG	NEG	NEG	3518	NEG	28998	661	156	NEG	3565	NEG
14/3/2013	21/3/2013	25/3/2013	28/3/2013	1/4/2013	4/4/2013	8/4/2013	11/4/2013		18/4/2013	22/4/2013
NEG	NEG	NEG	35799	547		2040	10349		1928	NEG

Wk 9			Wk 10			Wk 11			Wk 12		
25/9/2012	29/9/2012	1/10/2012	4/10/2012	8/10/2012	11/10/2012	15/10/2012	22/10/2012				
30722	8114	18673	854	NEG	NEG	NEG	NEG				
1/10/2012	4/10/2012	9/10/2012	11/10/2012	15/10/2012	18/10/2012	22/10/2012	25/10/2012				
NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG				
1/10/2012	4/10/2012	8/10/2012	11/10/2012	15/10/2012	18/10/2012						
NEG	NEG	NEG	NEG	NEG	NEG						
11/10/2012	15/10/2012	18/10/2012									
NEG	NEG	NEG									
18/10/2012		25/10/2012	29/10/2012	1/11/2012	5/11/2012	8/11/2012					
NEG		NEG	NEG	NEG	NEG	NEG					
22/10/2012	25/10/2012	29/10/2012	1/11/2012	5/11/2012		15/11/2012					
4417	8340		NEG	490		NEG					
29/10/2012	1/11/2012	5/11/2012	8/11/2012	12/11/2012	15/11/2012	19/11/2012	22/11/2012				
NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG				
8/11/2012	12/11/2012	15/11/2012	19/11/2012	22/11/2012	26/11/2012	29/11/2012	3/12/2012				
2165	173	NEG	166	NEG	164	NEG	NEG				
22/11/2012	26/11/2012	29/11/2012	3/12/2012								
NEG		NEG	NEG								
22/11/2012	26/11/2012	29/11/2012									
6783	1495	NEG									
10/12/2012		17/12/2012			9/1/2013						
NEG		NEG			NEG						
17/12/2012	20/12/2012	24/12/2012	27/12/2012	31/12/2012	3/1/2013						
1546	NEG	NEG	NEG	NEG	NEG						
	7/1/2013		14/1/2013	17/1/2013	21/1/2013				28/1/2013		
	113		NEG	NEG	NEG				NEG		
14/1/2013	17/1/2013	21/1/2013	24/1/2013				7/2/2013				
NEG	NEG	NEG	NEG			NEG					
28/1/2013	31/1/2013	4/2/2013	7/2/2013	11/2/2013	14/2/2013						
NEG	NEG	NEG	NEG	NEG	NEG						
7/3/2013	11/3/2013	14/3/2013	18/3/2013		25/3/2013						
24616	NEG	NEG	NEG								

14/2/2013	18/2/2013						
NEG	NEG						
28/2/2013							
NEG							
4/3/2013	7/3/2013	11/3/2013	14/3/2013	18/3/2013	21/3/2013	25/3/2013	28/3/2013
2334	1163	2217	722	10705	5838	NEG	83
7/3/2013	11/3/2013		18/3/2013		25/3/2013	1/4/2013	4/4/2013
324	8202		2121		NEG	1716	NEG
14/3/2013	18/3/2013	21/3/2013	25/3/2013	28/3/2013	1/4/2013	4/4/2013	8/4/2013
93871	46179	19321	14063	NEG	NEG	NEG	NEG
4/4/2013	8/4/2013	11/4/2013	15/4/2013	18/4/2013	22/4/2013	25/4/2013	29/4/2013
NEG	2263	NEG	NEG	NEG	NEG	NEG	NEG
25/4/2013	29/4/2013		6/5/2013				
151	362		NEG				
15/4/2013	18/4/2013	22/4/2013	25/4/2013	29/4/2013	2/5/2013	6/5/2013	9/5/2013
NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
25/4/2013	29/4/2013	2/5/2013	6/5/2013		13/5/2013		20/5/2013
NEG	NEG	NEG	NEG				