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Exploring Torque Teno Virus-Host Immune Interactions

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Contents

List of abbreviations

TTV	Torque teno Virus
TTMV	Torque teno mini virus
TTMDV	Torque teno midi virus
ICTV	International Committee on Taxonomy of Viruses
AV	Anelloviruses
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
ssDNA	single-stranded DNA
ORF	Open Reading Frames
IR	Intergenic Regions
HVR	Hypervariable region
UTR	Untranslated Region
TAIP	TTV-derived-apoptosis-inducing-protein
RCR	Rolling Circle Mechanism
vRep	viral replicative protein
IFNs	interferons
IL	interleukin
IFN-α	Interferon-alpha
IFN- β	Interferon-beta
IFN- γ	Interferon-gamma
IFN-λ	Interferon-lamda
IFN- ε	Interferon-epsilon
IFN-κ	Interferon-kappa
IFN-ω	Interferon-omega
IFN-δ	Interferon-sigma
JAK	Janus Kinases
STAT	Signal Transducers and Activators of Transcription
ISG	Interferon Stimulated Genes
NMI	N-myc and STAT interactor

miRNA	MicroRNA
mRNA	messenger RNA
PAMP	pathogen-associated molecular patterns
PRR	pattern recognition receptors
FAM	Fluorescein amidites
Ct	threshold cycle
cDNA	complimentary DNA
NA	Nucleic Acid
qRT–PCR	quantitative RT–PCR
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
CMV	cytomegalovirus
KT	kidney transplantation
DSA	donor-specific antibodies
SOT	solid organ transplantation
ESRD	end-stage renal disease
UTI	urinary track infection
uTTV	urinary TTV
pTTV	plasma TTV
MMF	mycophenolate
rATG	rabbit antithymocyte globulin
CMV	cytomegalovirus

Abstract

Background: Torque Teno Virus (TTV) is a ubiquitous component of human virome, not associated with any disease. TTV ability to establish persistent replication without overt clinical symptoms suggests a complex interaction with the host immune system.

Aims & Objectives: As its load increases when the immune system is compromised, such as in kidney transplant (KT) recipients, TTV load monitoring has been proposed as a method to assess immunosuppression. Simultaneously to investigate the interaction between TTV and host immune responses in the context of cytokine production may help to understand complex interaction with the host immune system.

Methodology: In this prospective study, TTV load was measured in plasma and urine samples from 42 KT recipients, immediately before KT and in the first 150 days after it. Furthermore, respiratory samples were also collected from 97 patients with respiratory changes caused by viruses to see the trend of TTV viral load and compare it with the cytokine expression also to see the trend across different age demographics.

Results: Data obtained suggest that TTV could be a relevant marker for evaluating immune status and could be used as a guide to predict the onset of infectious complications in the follow-up of KT recipients. Nevertheless, the study on cytokine profiling showed a significant positive correlation between TTV and the expression levels of key cytokines such as IFNL1 ($p = 0.0416$) and IFNL2 ($p = 0.0007$).

Conclusions: Since we observed no differences considering distance from transplantation, while we found a changing trend in days before viral infections, we suggest to consider changes over time in the same subjects, irrespective of time distance from transplantation. Other the other hand the nature of interaction between TTV and cytokines expression is at the moment unknown, this upregulation could contribute to the body defence against other pathogens by priming the immune system.

1. Introduction

Several viral agents other than the major known pathogenic viruses are found in clinical specimens. Referred to as the “human virome”, they are essential components in maintaining human health (Dal Lago, Brani et al. 2024). Torque Teno Virus (TTV) is a circular, single-stranded, nonenveloped, negatively polarized DNA virus measuring between 3.4 and 3.9 Kb in length and 30–32 nm in diameter. As a member of the family *Anelloviridae*, TTV belongs to the genus Alphatorquevirus (Spezia, Focosi et al. 2023).

No known disease has been associated with TTV, and it has been hypothesized that the entire human population is infected with TTV, often with multiple species simultaneously. Other hypotheses are that the virus remains in a persistent chronic form or that continuous reinfections occur when immune defences are lowered (Dal Lago, Brani et al. 2024), but not much is known about the interaction between TTV and the immune system. After the hypothesis of a possible pathogenic role for TTV was rejected, TTV attracted attention as a possible marker of immunosuppression (Focosi, Antonelli et al. 2016), since the viral load of TTV was inversely correlated with the competence of the immune system (Fernández-Ruiz, Albert et al. 2020).

The thesis includes the main objectives of the TTV project:

(1) To evaluate the presence of Torque Teno Virus as a biomarker of immune system function.

(2) To investigate the interaction between TTV and host immune responses in the context of cytokine production.

1.1. Introduction to Torque Teno Virus

1.1.1. TTV Taxonomy and Classification

Initially, it was believed that this new virus was a novel hepatitis virus, and it was named TT virus (TTV) after the initials of the patient in whom it was found. Previously, based on their genomic and other properties, TTVs were classified into the family Circoviridae (Miyata, Tsunoda et al. 1999, Mushahwar, Erker et al. 1999). But further genomic sequencing analysis of these viruses revealed that, despite exhibiting similar characteristics, these viruses do not show a significant homological relationship with the *Circoviridae* family. In 2004, TTV reformed its significance and became an abbreviation for torquetenovirus (derived from the Latin words torques and tenuis, meaning necklace and thin, respectively) when it complied with the International Committee on Taxonomy of Viruses (ICTV), following the rule that no official virus designation may be derived from a person's name. The discover of these

distinctions and other characteristics led to the creation of the novel *Anelloviridae* family, which derives from the Latin word anellus, which means "ring," to signify the circular genome (Biagini 2004, Biagini 2009).

According to a recent taxonomic update by the ICTV, the family *Anelloviridae* now includes 30 genera and 155 species, highlighting its considerable genetic diversity, which is believed to be primarily driven by extensive recombination. It appears that only eight genera have been identified as capable of infecting humans: *Alphatorquevirus* (TTV), *Betatorquevirus* (TT mini viruses - TTMV), *Gammatorquevirus* (TT midi viruses - TTMDV), and Hetorquevirus (Varsani, Opriessnig et al. 2021); more recently, Lamedtorquevirus, Memtorquevirus, Samektorquevirus, and Yodtorquevirus have also demonstrated human tropism (Laubscher and Anelloviridae). The prevailing view is that alpha, beta and gamma viruses are commensal components of the human virome (Moustafa, Xie et al. 2017) and are predominantly acquired during early childhood (Lim, Zhou et al. 2015). Furthermore, a remarkable trend of increasing TTV genomic diversity with age has been demonstrated (Laubscher, Hartley et al. 2022) although it is currently unknown whether this trend will continue into adulthood and how long such diversity will be maintained, nor is the possibility of being co-infected with a variety of different AV species and genotypes within the same genus (Reyes, Spezia et al. 2024). The discovery of viral diversity and the identification and characterization of non-human AVs, which are particularly important for understanding viral evolutionary dynamics, have been profoundly impacted by the advent of high-throughput sequencing (HTS) technologies and metagenomic protocols.

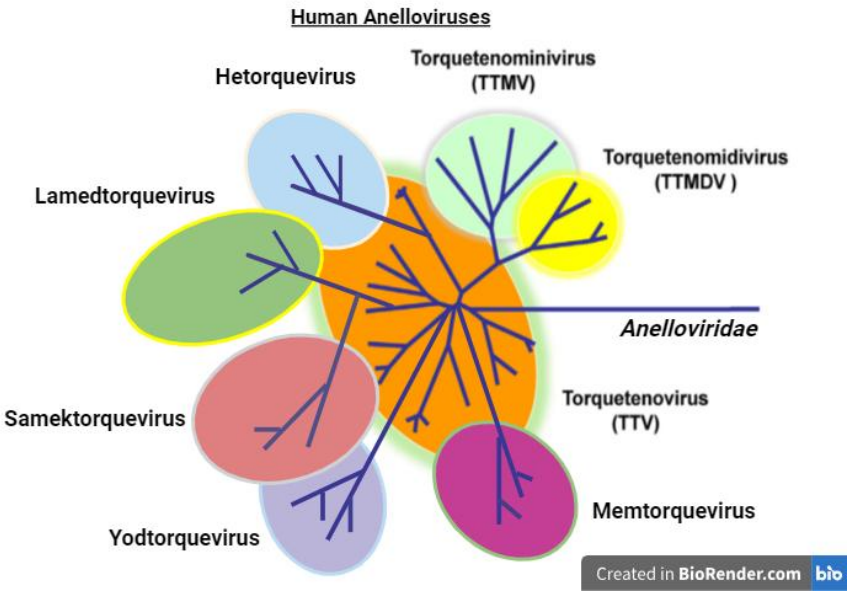


Figure 1.1: schematic representation of viral species of Anelloviridea: *Anelloviridae* now includes 30 genera and 155 species. Out of Eight Human Anelloviruses, three commonly infect humans: *Alphatorquevirus*, *Betatorquevirus*, and *Gammatorquevirus.* Modified from (Spezia, Focosi et al. 2023).

1.1.2. Genomic characterizations of TTV

The viral genome consists of coding and non-coding regions of 2.6kb and 1.2kb, respectively (Sabbaghian, Gheitasi et al. 2024). The non-coding area of the genome has a GC-rich conserved region (which is around 113 nt long and covers about one third of it), a promoter, and transcriptional enhancer elements (Okamoto, Nishizawa et al. 1998, Miyata, Tsunoda et al. 1999, Bostan, Amen et al. 2013). The coding region has a TATA box and a poly-A sequence in which overlapping open reading frames (ORF) in all three frames are situated (Erker, Leary et al. 1999, Hijikata, Takahashi et al. 1999, Miyata, Tsunoda et al. 1999). By alternative splicing means, TTV produced three mRNA species with length of 3.0 kb 1.2 kb and 1.0 kb, respectively (Miyata, Tsunoda et al. 1999). Further splicing creates two or more ORFs (ORF2/2, ORF1/1, ORF2/3, and ORF1/2) from the second (1.2kb) and third (1.0 kb) mRNA species (Timmerman, Schönert et al. 2024). The existence of extra potential ORFs that may encode less-known proteins and their function are yet to be deciphered (Kamahora, Hino et al. 2000, Spezia, Focosi et al. 2023). The general genomic organization is shown in Figure 1.2.

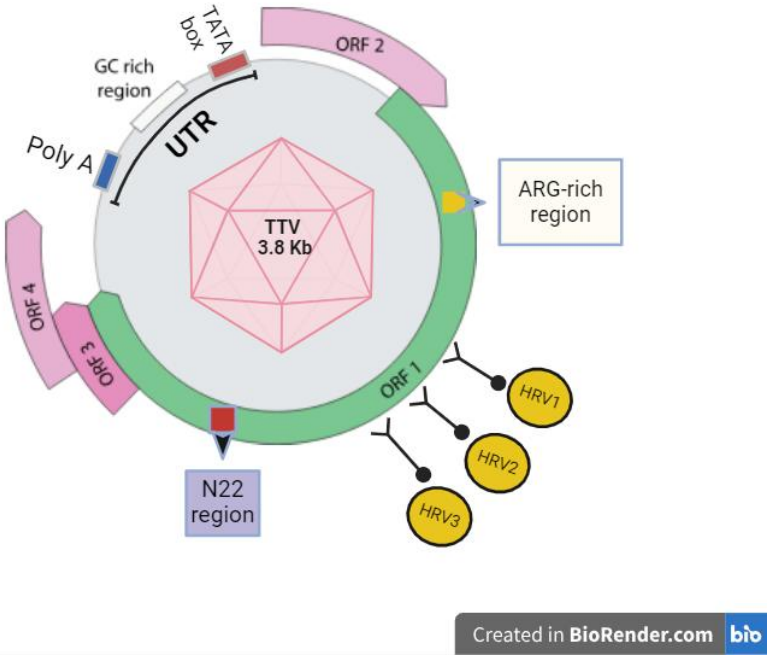


Figure 1.2: **Schematic representation of TTV genome structure**. TTV: torque teno virus; ORF: open reading frame; HVR: hypervariable region; UTR: untranslated region.

1.1.3. Genotypes & geographical distribution of TTV

TTV is now considered ubiquitous, to date, seven genotypes have been isolated (Hsiao, Wang et al. 2021) with a prevalence that varies by geographical area and can exceed 90% of the adult population (Nishizawa, Okamoto et al. 1997, Hino and Miyata 2007, Okamoto 2009). Genogroups 1, 2, 3, and 4 appear to be widely spread worldwide. However, the prevalence of genogroup 5 has not been fully confirmed. Genogroup, I comprise 1-5 TTV spices. Genogroup II (TTV 6-8), genogroup II (TTV 13-24), genogroup IV (TTV 25-29) and genogroup V include TTV 9-12 species. A few subtypes (1a, 1b, 2a, 2b) of TTV have also been identified (Reyes, Spezia et al. 2024).

Group 1 (G1) and group 2 (G2) TTV prevalent among hepatic disease patients and blood donors in the USA and Italy, whereas G4 is also recorded but is less frequent (Colombatto, Brunetto et al. 1999, Haramoto, Katayama et al. 2008, Bostan 2013). The map (fig. 3) highlighted the range of prevalence rates in the following areas:

Africa: 17% to 84% (Smuts and Tucker 2003, Pujol, Mejías et al. 2005, Hafez, Shaarawy et al. 2007).

Asia: 6.67% to 100% with specific mentions of high prevalence in Taiwan (Hsiao, Wang et al. 2016), Iran (Izadi, Samarbafzadeh et al. 2016), Pakistan (Hussain, Manzoor et al. 2012), and China, and lower rates in Thailand has been detected (Urwijitaroon, Barusrux et al. 2007).

Middle East: High prevalence in Qatar with 84.9%-90.75% (AbuOdeh, Al-Mawlawi et al. 2015), Saudi Arabia 42.9% (El-Taher, Fouad et al. 2015) and Turkey has been identified (Kalkan, Ozdarendeli et al. 2005).

Europe: 50.4% to 95% notably high in Russia (Vasilyev, Trofimov et al. 2009), lower in the Czech Republic (Saláková, Němeček et al. 2004).

North America (Canada) (Brassard, Gagné et al. 2015) 38.8% in diarrheal patients, and in South America 46% to 100% such as in Brazil (Mazzola, Saito et al. 2015) and Uruguay (Cancela, Ramos et al. 2016).

Figure 1.3: Prevalence of TTV: Distribution of torque teno virus. In orange: evidence of TTV presence described in the literature. In grey: no data.

1.1.4. TTV identification in human tissue

Infections in humans have been shown to occur at an early age (Kaczorowska, Cicilionytė et al. 2022) and have been identified in nearly every human tissue (Leppik, Gunst et al. 2007, Focosi, Antonelli et al. 2016, Blatter, Sweet et al. 2018), consistent with the lymphocytes being the primary site of AV replication (Leppik, Gunst et al. 2007). Interestingly, the virus load appears to be controlled by the immune system because virus levels increase with the level of host immunosuppression (Focosi, Antonelli et al. 2016, Blatter, Sweet et al. 2018).

Furthermore, it has been suggested that AVs positively affect human health by shaping immunity during early development (Pescarmona, Mouton et al. 2021) and are now considered part of the 'healthy human virome' (Arze, Springer et al. 2021, Koonin, Dolja et al. 2021), likely as a result of an extensive coevolution of AVs with their mammalian hosts (Fahsbender, Burns et al. 2017, Crane, Goebel et al. 2018, de Souza, Fumagalli et al. 2018, Webb, Rakibuzzaman et al. 2020). Numerous studies have suggested horizontal and vertical routes of TTV transmission. Horizontal transmission includes parenteral, fecal-oral, and sexual. Vertical transmission includes the possible passage of virus from mother to fetus during pregnancy and breastfeeding (figure 1.4) (Tyschik, Shcherbakova et al. 2017)

Nevertheless, the possibility of vertical transmission has been proposed, umbilical cord blood has been found to be negative for alphatorquevirus DNA, which has led to the conclusion that transplacental transmission of AVs is unlikely (Tyschik, Shcherbakova et al. 2017). It can be

hypothesized that AVs may be transmitted by breastfeeding, as the presence of AV DNA has been identified in breast milk (Schröter, Polywka et al. 2000). However, no association was observed between the infant's breastfeeding status and AV richness (Beller, Deboutte et al. 2022).

It is noteworthy that some studies have identified alphatorquevirus DNA in blood as early as the second month of life and in stool samples in the first months of life, suggesting replication in the gut at a very young age (Lim, Zhou et al. 2015). The peak of AV abundance in the gut was found between the sixth and twelfth months of life, after which the abundance decreases (Taboada, Morán et al. 2021). The same is probably true for the AV virome in the blood (anellome) and the early-life dynamics of anellome may contribute to the maturation of children's immune systems.

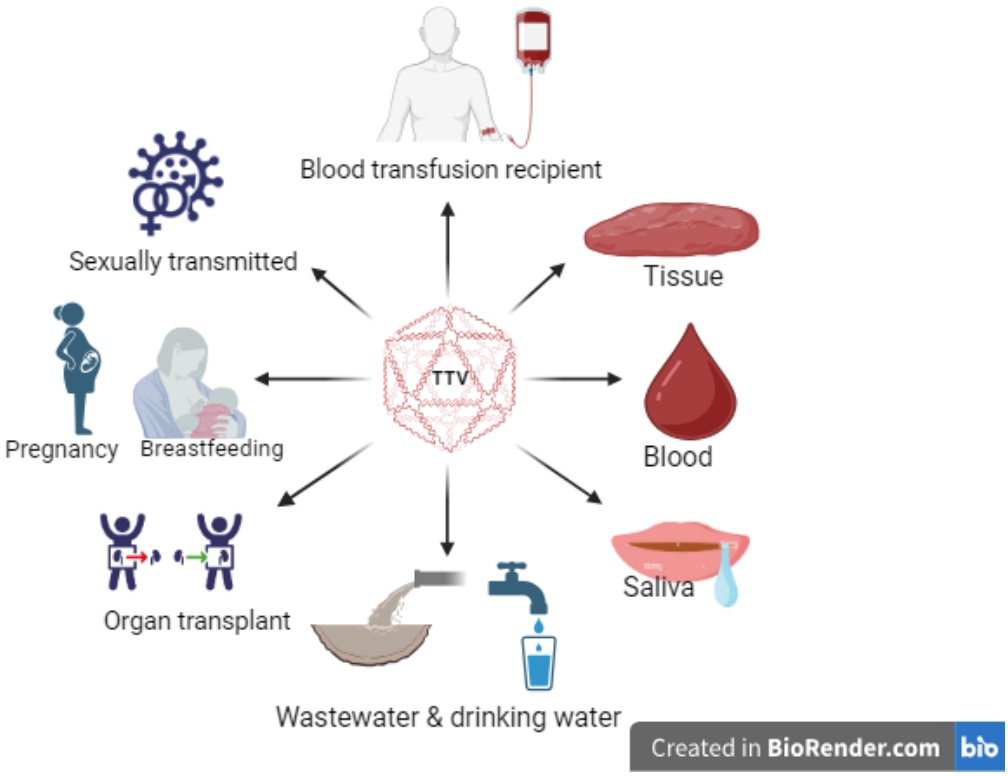


Figure 1.4: TTV transmission route.

1.1.5. TTV replication

TTV showed a wide range of host cell tropisms. Its DNA has been found in various tissues and body fluids (breast milk, serum, synovial fluid, peripheral blood, feces, saliva, and bile juices), except in red blood cells and platelets (Bostan, Amen et al. 2013). Since neither an animal

model nor a viral culture system are available, our understanding of the mechanism of replication is limited (Kaczorowska, Timmerman et al. 2023).

In-vivo replication system: A little is known about TTV life cycle and replication processes because of their analogy to other ssDNA viruses (Taylor, Keeler et al. 2022, Spezia, Focosi et al. 2023). Most of these DNA viruses rely on the host cell's replication machinery for their reproduction. TTV is thought to utilize host cellular polymerases rather than encoding a DNA polymerase for replication (Kaczorowska, Timmerman et al. 2023, Sabbaghian, Gheitasi et al. 2024). This idea was supported by treating TTV (HEL32 isolate, also called TTV 3) with a drug called aphidicolin (a medication that inhibits cellular DNA polymerase function), resulting in no replication of TTV, thus demonstrating that TTV uses host cellular replication machinery for DNA replication (Kakkola, Hedman et al. 2009). Based on similarities with other circular single-stranded DNA viruses, it is assumed that TTV could use the rolling circle mechanism. An overview of rolling circle replication (RCR) is given below, in figure 1.5. All single-stranded DNA (ssDNA) viruses that encode a viral replicative protein (vRep) possess highly conserved nick sites and similar rolling circle replication (RCR) motifs, which are crucial for DNA binding and cleavage (Rosario, Duffy et al. 2012). To make receptor-ligand interactions, TTV-like circoviruses produce replication-associated proteins with specific Rep-motifs that bind to the replication initiation site. TTV genomic part ORF1 ostensibly contains similar Rep-motifs (Erker, Leary et al. 1999, Kakkola, Hedman et al. 2009). TTV virion assembly and release strategy from the producer cells are still unknown. However, according to a new study, TTV particles are contained within circulating extracellular vesicles, providing new routes for progeny virions to leave the body (Spezia, Focosi et al. 2023).

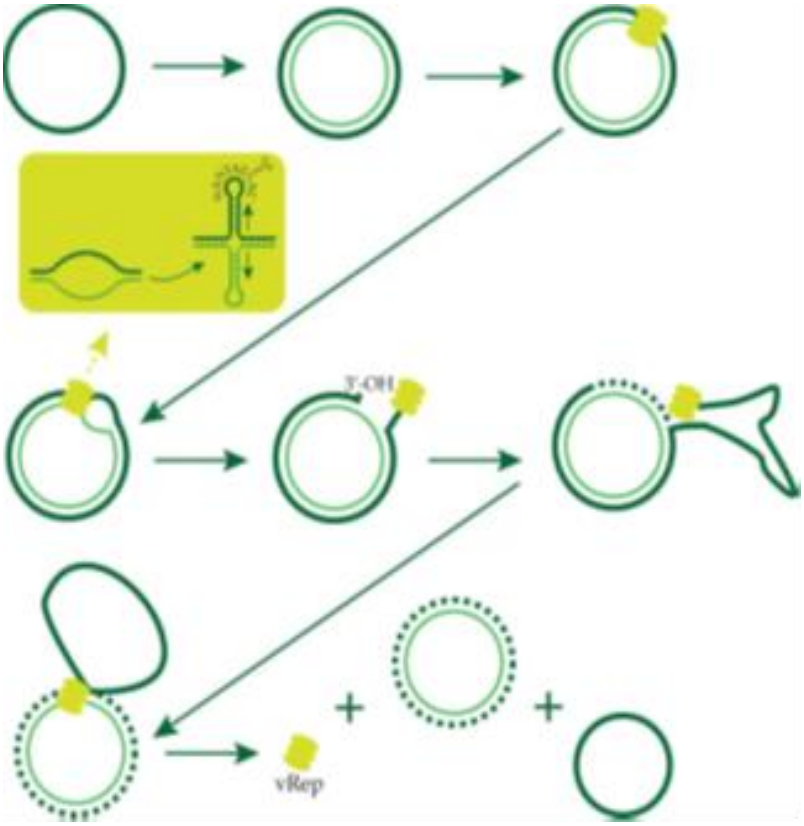


Figure 1.5. overview of rolling circle replication (RCR): The viral ssDNA is converted into a double-stranded replicative form using host factors. During initiation, the viral replication initiator protein (vRep) binds to the replicative form near the origin of replication, likely causing partial melting of the dsDNA. This melting may result in a cruciform structure that exposes the nick site of the virion strand (thick line) in its single-stranded form. vRep introduces a nick between the thymine at position 7 and the adenine at position 8. This nick serves as a primer for leading-strand synthesis by host DNA polymerases, with the 5' end covalently linked to vRep. During elongation, the old virion strand (thick line) is displaced by the newly synthesized positive strand (dashed line). Rolling circle replication (RCR) concludes when vRep catalyses a joining reaction between the ends of the newly synthesized positive strand, releasing the old virion strand (Rosario, Duffy et al. 2012).

In-vitro replication system: to date, a true cell culture system that sustains effective TTV replication is not yet obtainable. This leads to delays in the investigation of TTV gene replication and gene expression. The comprehensive transcriptional profile of TTV was described by transfecting a clone of TTV genotype 6 in 293 human cells. Furthermore, the extra splice variants were exhibited when genotype 5 isolates tth7 and tth8 were transfected into L428 Hodgkin's lymphoma cells (Leppik, Gunst et al. 2007). In infected bone marrow cells, three species of TTV mRNAs ranging in size from 2.8–3.0 kb, 1.2 kb, and 1.0 kb have been detected. The same species has been identified in transfected Cos-1, 293 and L428 cells (Kakkola, Hedman et al. 2009). However, maintaining the TTV viral load for a longer period in the cell lines is still unachievable.

1.1.6. TTV and immune system

After the hypothesis of a possible pathogenic role for TTV was rejected, TTV attracted attention as a possible marker of immunosuppression (Focosi, Antonelli et al. 2016) since the viral load of TTV was inversely correlated with the competence of the immune system (Fernández-Ruiz, Albert et al. 2020). In fact, hosts with compromised immune function due to a variety of etiologies, including sepsis (Mallet, Diouf et al. 2021), HIV infection (Madsen, Eugen-Olsen et al. 2002, Thom and Petrik 2007, Nasser, de Oliveira et al. 2009, Schmidt, Jensen et al. 2021), cancer (Zhong, Yeo et al. 2001, Starzer, Mayer et al. 2021), bone marrow (Kanda, Tanaka et al. 1999, Masouridi-Levrat, Pradier et al. 2016, Albert, Solano et al. 2018), and solid organ transplantation (SOT) have, on average, higher TTV viral loads than the general population.

Kidney transplantation (KT) is the gold standard for the treatment of end-stage renal disease (ESRD). The immunosuppressive drug regimen plays a key role in KT, reducing rejection rates and preventing the formation of donor-specific antibodies (DSA). However, a high immunosuppressive burden increases the patient's susceptibility to both opportunistic infections (Fishman 2007) and cancer (Ying, Shi et al. 2020). As immune status plays a key role in determining K outcomes, estimating the optimal immunosuppressive load is one of the major challenges in the clinical management of SOT recipients (Shrestha 2017). The current approach is based, for some immunosuppressants, on monitoring the plasma drug load over time. Although this approach has contributed to increased graft and patient survival over the past 20 years, it has inherent limitations as there is no universal optimal dose because several factors play a role in determining the therapeutic window, including the association with other immunosuppressive agents, time since KT, and immunologic risk. In addition, maintaining the therapeutic window is complicated by the large interindividual pharmacokinetic differences in the metabolism of immunosuppressive drugs (Andrews, Li et al. 2017) and it does not appear to play a role in the long-term prediction and prevention of chronic rejection and adverse events (Meier-Kriesche, Schold et al. 2004).

The infection rate after SOT varies over time; it is highest in the first three months after transplantation and then gradually decreases, and immunosuppressive therapy is the main risk factor for infectious complications (Fishman 2007, Cross, Berry et al. 2008, Mella, Mariano et al. 2022). Among the most important pathogenic viruses in the immunocompromised host are BK polyomavirus and cytomegalovirus (CMV) (Lumbreras, Manuel et al. 2014, Requião-Moura, Matos et al. 2015). Bacterial infections also affect KT recipients more frequently than

the general population: UTIs are particularly common, with the most relevant pathogens being Enterococcus faecalis, Escherichia coli, Pseudomonas aeruginosa, and Klebsiella pneumoniae.

Infections in these patients are difficult to detect and treat for several reasons, including the fact that symptoms are often masked by the administration of immunosuppressive therapy, that a greater number of different pathogens may be responsible for the infection (including opportunistic pathogens), and that there may be interactions between antimicrobial and immunosuppressive therapy. Early detection is of paramount importance to promptly initiate treatment and prevent further complications, including graft loss and death (Fishman 2007, Requião-Moura, Matos et al. 2015).

In conclusion, there is a need in the management of SOT to identify a marker that overcomes the inherent limitations of the currently used methods for assessing the level of immunosuppression and predicting infection, and that could be used independently or in combination with them (Wieland, Olbricht et al. 2010). Ideally, this marker should be able to detect both excessive and inadequate immunosuppression, be standardized, and be easy to implement in routine follow-ups (Jaksch, Kundi et al. 2018). Currently, several markers have been proposed, mostly based on specific elements that play different roles in the immune response, but none of them have been validated and actively used in clinical practice. These markers provide a limited picture of immune status due to test specificity; the direction that research is attempting to take is to instead use a marker that would reflect overall immune function. Although TTV plasma levels are mainly controlled by T cells, the control is also partly mediated by other components of the immune response, reflecting T lymphocyte function and overall host immune function (Jaksch, Görzer et al. 2022).

Immediately following SOT, an increase in total human viral load and genotypic diversity is observed (De Vlaminck, Khush et al. 2013, Kulifaj, Tilloy et al. 2020). Specifically, anelloviruses become predominant and make up 94% of the human virome (De Vlaminck, Khush et al. 2013) and TTV seropositivity rises to 100% (Querido, Martins et al. 2023). Assessment of TTV viremia appears to better characterize a patient's immunosuppression status than current methods, as viral load appears to be associated with the dose and type of immunosuppressive drug (Ling, Xiong et al. 2012, Focosi, Maggi et al. 2014, Focosi, Antonelli et al. 2016), but also with both short- and long-term adverse effects, such as acute graft rejection, higher infection rate, chronic graft rejection, and death (Blatter, Sweet et al. 2018,

Strassl, Schiemann et al. 2018, Doberer, Schiemann et al. 2020, Gore, Gomes-Neto et al. 2020, Van Rijn, Roos et al. 2023).

The precise range of TTV concentrations at which optimal immune function is achieved remains unclear. While several studies have identified different cut-offs at different time points, there is no conventionally accepted cutoff (Maggi, Focosi et al. 2018). Although the role of plasma (pTTV) in SOT recipients has been investigated in several studies, there are currently no data on the potential role of monitoring urinary TTV load (uTTV) in these immunosuppressed patients. TTV levels also appear to vary according to pre-transplant characteristics of KT recipients (such as age at transplantation, gender, pre-KT dialysis (Takemoto, Okubo et al. 2015, Balzer, Pankow et al. 2020, Ngamvichchukorn, Ruengorn et al. 2022), and underlying disease (Giacconi, Maggi et al. 2018)), although their role is controversial. Most of the available studies show an association between age and TTV viral load (Giacconi, Maggi et al. 2020, Tepel, Nagarajah et al. 2022), which can be explained by the fact that in the elderly there is a reduction in the efficiency of the immune system, known as immunosenescence (Cleveland and Devlin 1988, Focosi, Macera et al. 2015).

1.2. TTV and Its Relationship to Interferons

TTV ability to establish persistent replication without overt clinical symptoms suggests a complex interaction with the host immune system, particularly involving innate immune responses. Many studies have investigated that TTV may have immunomodulatory effects, potentially influencing cytokine production and the immune response to other viral infections (Moen, Sagedal et al. 2003). The idea that ORF2 protein aids in the virus's adaptation to the host environment is strengthened by its capacity to block both canonical and noncanonical NF-κB pathways (Zheng, Ye et al. 2007). When this pathway is inhibited, inflammatory cytokines (IL6 and IL8) are produced at a lower rate and gene translation is disturbed (Sabbaghian, Gheitasi et al. 2024).

On the contrary, it is also known that Anelloviruses are susceptible to interferon. In vitro interferon-gamma (IFN- γ) treatment of a TTV-transfected Hodgkin lymphoma cell line L428 and a TTV-transfected kidney cell line 293 revealed inhibited TTV DNA replication (de Villiers, Kimmel et al. 2009). In addition, people treated with Interferon-alpha (IFN-α) due to a hepatitis C virus infection generally showed decreasing TTV concentrations in blood (Nishizawa, Tanaka et al. 2000, Maggi, Pistello et al. 2001). In another study, messenger RNA (mRNA) expression of a few of anti-viral proteins, including MxA, 2'5'OAS, antiapoptotic

protein, cytokines IL-28, IL-29, and IFN-α, IFN- β (Interferon-beta), has been studied in BJAB and Dohh2 B cell lines. The findings depicted that MxA may act as a protection against ssDNA viruses because it is not expressed in the Raji cell line, which is susceptible to TTV infection (Garbuglia, Grasso et al. 2007).

1.2.1. Interferons and Interferon signalling pathway

Interferons: Interferons (IFNs) are a group of signaling proteins that are produced and released by host cells in response to the presence of viruses, bacteria, parasites, and tumor cells. They are part of the body natural defense mechanism against pathogens (Zhang and An 2007).They are classified into three main types: Type I, Type II, and Type III, each with distinct roles in the immune system.

Type I Interferons: Type I IFNs consist of seven classes: Interferon- alpha (IFN-α), IFN- β (beta), IFN- ε (epsilon), IFN-κ (kappa), IFN-ω (omega), IFN-τ, and IFN-δ (sigma). In addition, four IFN-like cytokines such as limitin (found only in mice), interleukin-28A (IL-28A), IL-28B, and IL-29 found in humans and other mammals have been reported. These are produced by almost all cell types in response to viral infections. They activate antiviral genes, enhance antigen presentation, and stimulate natural killer (NK) cells and T cells, thereby orchestrating a robust antiviral response (Pestka, Krause et al. 2004, McNab, Mayer-Barber et al. 2015).

Type II Interferons (IFN-γ): there is only one type II IFN is IFN-γ. It is mainly produced by T cells and NK cells, IFN-γ is critical for adaptive immunity. It enhances macrophage activity and promotes the differentiation of T helper cells, playing a key role in controlling infections (Lee and Ashkar 2018).

Type III Interferons (IFN-λ): These include IFN-λ1 (IL-29), IFN-λ2 (IL-28A), and IFN-λ3 (IL-28B), which have antiviral effects similar to Type I interferons but act primarily on epithelial cells. They are particularly important in mucosal immunity, providing a first line of defence in the respiratory and gastrointestinal tracts (Zanoni, Granucci et al. 2017).

Interferons signalling pathway: an inflammatory process triggers upon viral-cells infection, which results in secretion of interferons, among other molecules, such as, prostaglandins and interleukins (Zhang and An 2007)**.** All Interferons bind to their specific receptor on the cell surface and start a signalling surg through the proteins of Janus Kinases (JAK) and Signal Transducers and Activators of Transcription (STAT) family members, that initiate the transcription of Interferon Stimulated Genes (ISGs) that inhibit the replication of different

types of viruses as shown in figure 1.6 (Pestka, Krause et al. 2004, Ahmad, Valverde et al. 2020, Sposito, Broggi et al. 2021).

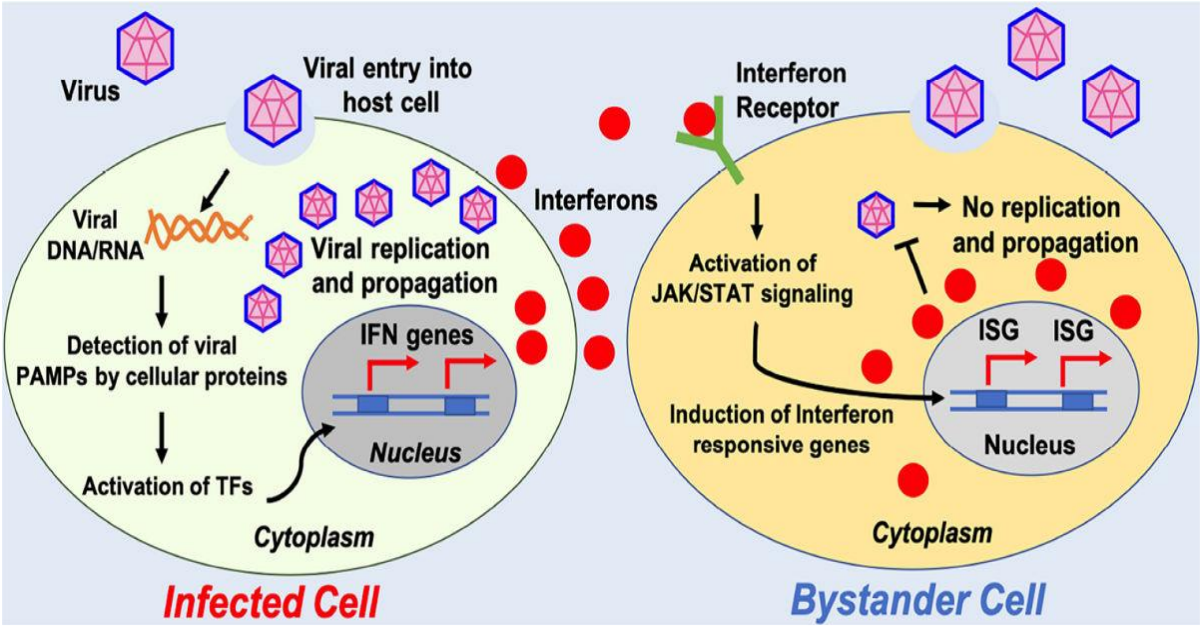


Figure 1.6. Activation of the interferon pathway can effectively restrict and eliminate viruses. The interferon pathway is part of the innate immune response activated by virus-infected cells. When virus-derived factors or replication intermediates (such as nucleic acids) are recognized, they trigger a host signalling cascade that leads to the production of interferons. These secreted molecules function in both paracrine and autocrine manners to activate neighbouring cells, inducing an antiviral state (Ahmad, Valverde et al. 2020).

1.2.2. Viral miRNA suppression of interferon signalling

A canonical and non-canonical interferon signaling pathways involved in the host antiviral response but in certain viruses, in particular, DNA viruses produce miRNAs that have ability to modify the host interferon pathway, by inhibition of specific host transcripts that act as critical regulators of the interferon response and the immune response as a whole. MicroRNAs (miRNAs) are small noncoding RNA molecules, typically 21 to 25 nucleotides in length, that regulate gene expression post-transcriptionally by base-pairing with their target mRNAs. (Ahmad, Valverde et al. 2020). TTV uses its miRNA, TTVtth8-miR-T1, to weaken the host's immune defense by targeting NMI, a key regulator in the JAK-STAT pathway. TTV tth8 miRNAs are generated by the canonical host miRNA biogenesis pathway. This pathway is crucial for the antiviral response, as it activates genes to fight infections. TTVtth8-miR-T1 binds to the NMI mRNA, reducing its protein levels and thus impairing the JAK-STAT signaling. By downregulating NMI, TTV effectively dampens the immune response, allowing the virus to persist undetected in the host (Kincaid, Burke et al. 2013).

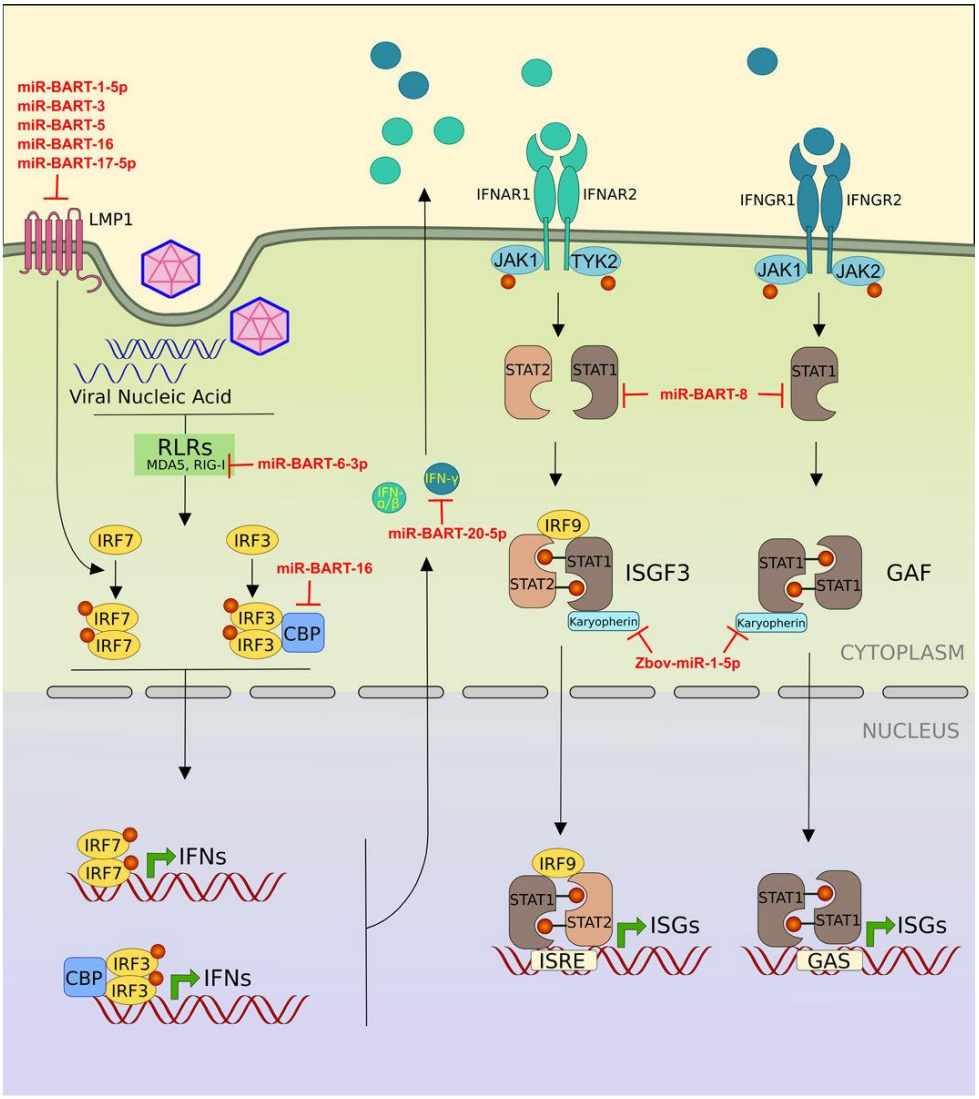


Figure 1.7. Viral miRNAs target genes involved in the interferon signalling pathway. Infected cells detect viral pathogen-associated molecular patterns (PAMP) through various pattern recognition receptors (PRR) like RIG-I, which triggers the secretion of interferons. Once released, interferons bind to their receptors, stimulating the expression of numerous interferon-stimulated genes (ISGs) that work together to establish an antiviral state within the cell. Various viral miRNAs (indicated in red) inhibit different genes within the interferon pathway (Ahmad, Valverde et al. 2020).

2. Aims of the Study

2.1. Validation of TTV as a marker of immune status in KT recipients:

- The aim of our study is to validate TTV as a marker of immune status in KT recipients.
- To evaluate TTV plasma and urine kinetics from pre-transplant to 150 days after KT.
- To investigate the relationship between inter-individual differences in viral load and clinical factors or recipient characteristics at baseline.
- To evaluate the associations between TTV and infectious events.

2.2. Correlation Between Torque Teno Virus (TTV) and Host Immune Responses:

- To find the correlation between TTV and cytokines production.
- To find the pattern of cytokines production with respect to TTV load in the host.
- To find the correlation of cytokines in respiratory samples with respect to age.

3. Materials and Methods

3.1. Validation of TTV as a marker of immune status in KT recipients

(1) Experimental model:

A total of 42 consecutive patients who underwent kidney transplantation at the Transplant Centre of Ospedale di Circolo and Fondazione Macchi ASST Sette Laghi in Varese, Italy, between September 2022 and March 2023 were enrolled in this prospective study. All enrolled KT recipients fulfilled the following inclusion criteria: (a) KT was performed in our center, (b) regular follow-up was performed in our outpatient clinic for at least the first 3 months after KT, (c) only patients initially treated with maintenance tacrolimus (FK), mycophenolate (MMF), and steroids (CS) were included. Exclusion criteria were: (a) transplantation and/or initial treatment in another center and (b) maintenance treatment other than allogeneic hematopoietic cell transplantation. Ethical approval for this study was obtained from the Ethical Committee of University of Insubria—approval number 74724.

Post-transplant care was provided according to the standards of our center and KDIGO 2009 guidelines. All patients received peri-HCT induction therapy selected according to donor and recipient risk factors: Basiliximab and steroids were administered to all patients, while only high-risk patients were additionally treated with rATG. MMF was started on the first post-KT day, while FK was usually started on days 4–5. During follow-up, plasma tacrolimus trough levels were monitored, and tacrolimus doses were adjusted if out of range.

In accordance with our center's standard of care, no protocol graft biopsies were performed, and no alloantibodies were measured. No patient showed signs of acute rejection during follow-up. Allograft rejection, defined as graft dysfunction and failure due to the recipient's immune response to the genetically different graft, is diagnosed by an initial clinical evaluation and laboratory tests designed to assess renal function, creatinine, and urea in the first place. If rejection is suspected, donor-specific anti-HLA antibodies (DSA) are evaluated, and ultrasound and renal biopsy (the gold standard for certainty of diagnosis) are performed.

All patients were closely monitored for post-transplant infections. No patient was treated with CMV prophylaxis according to our center's standards, as CMV viral load monitoring is currently in place to inform preemptive treatment with antivirals. The CMV viral load above which antiviral therapy is given is 3000 copies/mL, and in case of rapid rise, therapy is started at lower values, 2000 copies/mL. All patients received trimethoprim/sulfamethoxazole chemoprophylaxis for the first six months after KT.

3.1.1. DNA extraction & quantification

Plasma and urine samples were collected before KT and at selected time points, weekly, or even more frequently in the immediate posttransplant period, during the first 100 days after KT. TTV levels were compared with clinical data from the patients' medical records, microbiological analysis, and blood tests (including trough FK levels, serum creatinine, and blood count). Plasma and urine samples were collected and stored at −80 ◦C until processed.

Automated DNA extraction was performed using BioerNPA-32P (Bioer Technology, Hangzhou, China) and MagaBio plus Virus DNA/RNA Purification Kit II (Bioer Technology). All samples were stored less than three hours at 4°C before extraction. After that the samples and the extraction kit were transported at room temperature. To prevent PCR contamination, isolation was performed in a separate DNA-extraction room (laboratory of microbiology). Following the purification kit manuals 10ul PK solution were taken and added in 1st and 7th column of the extraction plate. After that 200ul of patient samples were loaded according to their assigned position in the plate. The labelled plates were placed in a BIOER extraction machine. The purification procedure lasts for 37 minutes. After the completion of extraction 50ul of purified DNA were collected from column 5th and 11th respectively and stored at -20C until use.

Extracted DNA was quantified fluorometrically using Invitrogen (Waltham, MA, USA)—Qubit 4 according to the manufacturer instructions. The Qubit dsDNA BR Assay Kit has a sensitivity range between 0.1–120 ng. Following the protocol, reagents were diluted by the ratio 200:1 (buffer: fluorescent dye) respectively. 198ul of prepared solution were added and loaded 2ul of DNA samples into each labelled Qubit vials. And concentration was read using the Qubit 4.0 Fluorometer after a 2-5 minute of incubation and samples were stored at −80 ◦C.

3.1.2. Real-time PCR analysis

All samples were undergone for amplification process.

The extracted DNA was amplified by real-time PCR using TaKaRa Premix Ex Taq ™ (Takara Bio Group, San Jose, CA, USA) and Applied Biosystems Taqman universal PCR master mix (Thermo Fisher Scientific, Waltham, MA, USA). Primers and probe were designed from a portion of the untranslated region (UTR), which was found to be highly conserved among all TTV sequences available in the GenBank at the time of writing. The oligonucleotide sequences are as follows: AMTS (forward primer 5′-GTGCCGIAGGTGAGTTTA-3′, position 177-194), AMTAS (reverse primer 5′-AGCCCGGCCAGTCC-3′, position 226-239), and AMTPTU

(TaqMan probe 5′-TCAAGGGGCAATTCGGGCT-3′, position 205-223). The probe was labeled 6-carboxyfluorescein (FAM) and 6-carboxy-tetramethyl-rhodamine (TAMRA) at its 5′ and 3′ ends, respectively. Each run contained several negative controls (no template) as well as the reference template (positive control) at 10^1 to 10^6 copies/10 µL. Procedures for quantification of copy number and evaluation of intra- and inter-assay precision and reproducibility of the assay have been previously reported. The lower limit of sensitivity of the assay was 1.0×10^1 copies per mL of plasma or urine.

Table 3.2.1 PCR Mix proportions for single sample

	microliters (μL) x1
Master Mix	12.5
Forward primer	2.25
Reverse primer	2.25
Probe	0.25
Water	2.75

Table 3.2.3 TTV PCR thermal profile

Temperature	Time
50°C	2 mintues
95°C	2 mintues
95°C	1 mintues
60°C	20 seconds

3.2. TTV and its relationship to immune system

(2) Experimental model

A study was also carried out to find the correlation between TTV and the immune system. In this study, respiratory samples were collected from patients with respiratory changes caused by viruses to see the trend of TTV viral load and compare it with the cytokine expression also to see the trend across different age demographics. This study was carried out at the Microbiology Laboratory of the Ospedale di Circolo e Fondazione Macchi, ASST Sette Laghi. A total population of 97 respiratory samples were collected in order to find the correlation between TTV and cytokine production, and to find the pattern of cytokine production in relation to TTV load in patients. This sample population was divided into three main age groups: 1-18, 20-63 and 65-92 years. Out of ninety-six samples, 34 (1-18 years old), 31 (20-63 years old) and 21

subjects (65-92 years old) DNA samples were analysed for the presence of TTV and its correlation with interferons (INFL1, INFL2 and INFB1) and IL1B, IL6 cytokines.

For this pourpose, 97 respiratory samples for cytokine analysis were collected and processed for DNA extraction, quantification by Qubit and amplification by real-time PCR according to the protocol and conditions described above in section 3.1.1 & 3.1.2. All samples were stored at -80°C until further processing.

3.2.1. Cellular RNA extraction for cytokine analysis

The total RNA was extracted from respiratory specimens by using, ELITe InGenius and procedure was followed according to supplier's protocol and stored at -80°C until complimentary DNA (cDNA) synthesis. The ELITe InGenius extraction workflow is shown in Figure 3.2.1.

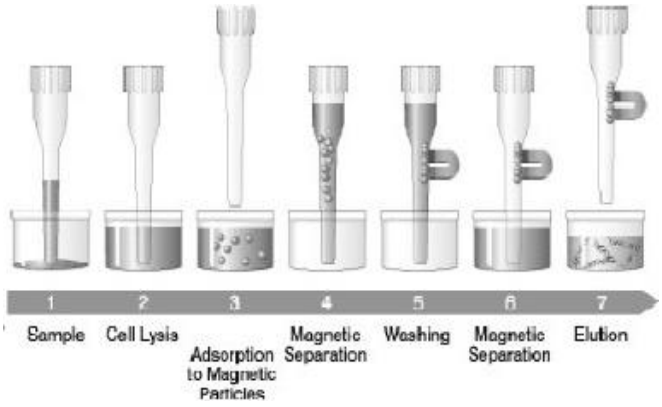


Figure 3.2.1: Extraction workflow

The samples also labelled with a unique barcode, preventing unwanted transpositions. The purified nucleic acids obtained from the extraction process are eluted and tubes caps were closed with the screw cap and sample were stored at -20°C or -70°C until cDNA synthesis.

3.2.2. cDNA synthesis

The extracted samples were then processed for cDNA synthesis by using Invitrogen™ SuperScript™ VILO™ cDNA Synthesis Kit. The Invitrogen™ SuperScript™ VILO™ cDNA Synthesis Kit provides the high temperature capability of SuperScript™ III Reverse Transcriptase in an optimized format for generating first-strand cDNA for use in real-time quantitative RT–PCR (qRT–PCR). This formulation provides enhanced cDNA synthesis efficiency and can be used with very low and very high amounts of input RNA (up to 2.5 µg total RNA in a 20-µL reaction), giving a linear response in message abundance as measured by

qPCR. The following protocol has been optimized for generating first-strand cDNA for use in two-step qRT–PCR. The reaction volume may be scaled as needed up to 100 µL. In our experiment, 20 µL reaction volume was used.

For a single reaction, combine the following components in a tube on ice as shown in table 3.2.1.

Table 3.2.1 recipe for a single reaction

Component	Quantity
5X VILO™ Reaction Mix	4 µL
10X SuperScript™ Enzyme Mix	2 µL
RNA (up to 2.5 µg)	x µL
DEPC-treated water	to 20 µL

For multiple reactions, a master mix was prepared without RNA. 6 µL of prepared master mix was added into labeled PCR tubes. Then 12 µL of extracted RNA samples were loaded into the tubes. After Gently mixing the tubes contents, all tubes were placed into thermocycler. The reaction was run on following thermocycler condition for cDNA synthesis.

Table 3.2.2 SuperScript™ VILO™ cDNA Synthesis temperature and time

Temperature	**Time**
25C	10 mintues
42C	60 mintues
85C	5 mintues

3.2.3. Cytokine Expression analysis

After cDNA synthesis, a 96 well dilution plate was made in which each single well corresponded to a triplicate of the sample. For a single sample, the following recipe was prepared (table 3.2.3).

Table 3.2.3 PCR Mix proportions for single sample

	microliters (µL) x1
Master Mix	2.5

Probe/gene	0.25
H2O	1.25

For 96 well plate, and qRT-PCR analysis was then carried out with TaqmanTM Fast Advanced Master Mix (Applied Biosystems Cat#4444963) by using specific TaqmanTM Gene Expression Assays from Thermo Fisher. Genes used for cytokine analysis are as follows:

- IFNL 1
- IFNL 2
- IFN B1
- IL-1B
- IL-6
- GAPDH

IFNL1 (Hs01050642_gH), IFNL2 (Hs04193047_gH), IFNB1 (Hs01077958_s1), IL1B (Hs01555410_m1) and IL6 (Hs00174131_m1) expression was assessed with respect to the housekeeping gene Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1). 12.2 µL of M mix was added into each well of column 1, 4, 7 and 10 of 95 wells plate. 3.5 µL of cDNA samples was added into column 1 and after mixing well, 5 µL was loaded to column 2 and then again took 5 µL of sample and loaded into column 3 (the sample run in triplicate manner). Repeated for 4, 7, and 10 columns.

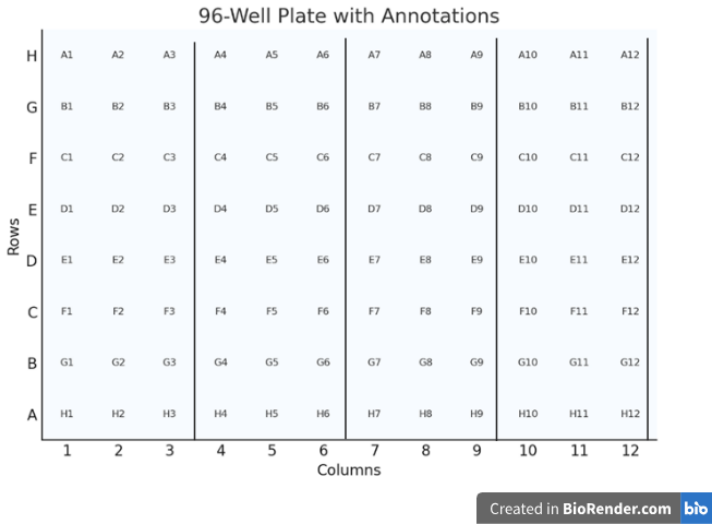


Figure 3.2.2 a 96-well plate prepared for cytokine analysis

All transcripts were tested in triplicate for each sample on QuantStudio 3 Real-Time PCR System (Thermo Fisher) at following PCR conditions.

Table 3.2.4 the PCR thermal profile

Temperature	Time
50°C	2 mintues
95°C	2 mintues
95°C	1 mintues
60°C	20 seconds

3.3. Statistical analysis

The post-transplantation kinetics of TTV were analyzed using the MEANS procedure of the SAS software, quantifying for each timepoint the mean plasma concentration of creatinine and FK and the plasma and urinary viral loads of TTV.

Descriptive statistics were performed to report the general characteristics of the population. To report an estimate of trends for the TTV DNA copies during time, a locally weighted regression has been used, using a scatterplot smoothing method that automatically determines the optimal smoothing parameter (PROC SGPLOT with LOESS statement in SAS). The relationship between clinical factors and plasma or urinary viral load of TTV was analyzed by multiple regression analysis, using baseline levels of pTTV or uTTV as the dependent variable and recipient's age at transplantation, recipient's sex, delayed graft function, years of dialysis, induction therapy, and maintenance therapy with FK, as the independent variables. Then, the same analyses were performed using baseline and follow up pTTV and uTTV levels as dependent variables and plasma creatinine, lymphocytes, leukocytes, tacrolimus trough levels and days since transplantation as independent variables. For these last analyses, the repeated subjects option was used in PROC GENMOD of the SAS software to take into account the repeated measurements performed in the same subjects. The analyses were then replicated, adding days since transplantation and variables found statistically significant associated with TTV levels in previous analyses, as covariates. Finally, the same analyses were performed using DGF or infection during follow-up as dependent and TTV as independent variables. We further performed a Cox regression analysis considering all covariates as time-dependent, and first incident infection occurred during follow-up, at the next measurement day, as event (PROC PHREG with count method for time-dependent covariates in SAS software). This method deals with the undue increase in statistical power of an analysis of each repeated measurement of TTV values in the same subjects. The variable TTV DNA copies have been log-transformed, using the formula ln (number of copies plus 1), to deal with the problem of

log(0) in case of negativity for TTV. The statistical analyses were performed using the SAS software 9.4 (v9.4, SAS Institute Inc., Cary, NC, USA) or by means of the GraphPad software Prism 9.

4. Results

4.1. Baseline Characteristics

The characteristics of the 42 enrolled patients are shown in Tables 4.1 and 4.2. The mean ± SD of the actual duration of follow-up was 103.4 ± 38.4 days.

Table 4.1 Population characteristics.

Variable	**N**	**Mean**	**SD**
Age (years)	42	54.7	12.1
Follow up days	42	104.9	38.5
Dialysis day	42	4.39	3.52

Table 4.2. Population characteristics. CMV: cytomegalovirus, rATG: rabbit antithymocyte globulin.

	N	**%**
Men	24/42	57.1
Kidney disease:		
Glomerular disease	13/42	28.6
Cystic disease (ADPKD)	8/42	19.0
Diabetic nephropathy	4/42	9.5
Hypertensive nephrosclerosis	4/42	9.5
Nephrectomy	5/42	11.9
Chronic pyelonephritis in VUR	3/42	7.1
Others	5/42	11.9
Positive CMV serostatus at baseline	37/42	88
Living donor transplant	8/42	19
Previous transplants	7/42	16.6
Double induction therapy (Basiliximab + rATG)	13/42	31.0
Delayed Graft Function (DGF)	14/42	33.3

The cohort of patients recruited had similar characteristics (e.g., age at transplantation, sex, and time on dialysis) to the population of KT recipients from the National Transplant Center. The most common underlying diseases we found in the recruited patients were glomerular diseases (28.6%) and cystic diseases (19%), followed by diabetic nephropathy, hypertensive nephrosclerosis, nephrectomy, chronic pyelonephritis in vesicoureteral reflux (VUR), and other syndromes. Most patients had a positive CMV serostatus at baseline (88%), received the organ from a cadaver donor (81%), and had never received a kidney transplant before (83%). More than half of the patients (69%) received basiliximab in the induction phase, while the remaining patients (31%) received double induction with basiliximab and thymoglobulin; Table 4.3

provides a detailed overview of the immunological characteristics of the patients. Finally, 14 subjects (33.3%) experienced a delayed graft function after transplant, defined as the need for substitutive therapy (dialysis) in 7 days after KT. These events demonstrated no correlation with pTTV and uTTV.

Table 4.3. Population immunological characteristics. PRA: panel-reactive antibody; MM: mismatch number.

Induction Therapy	**N**	**PRA% (Mean ± SD)**	**MM (Mean ± SD)**	**pTTV (Mean ± SD)**	**uTTV (Mean ± SD)**
Basiliximab + rATG	13	15 ± 0.13	6.3 ± 2.69	5.58 ± 2.09	3.16 ± 1.63
Basiliximab	29	8 ± 0.08	5.6 ± 2.16	5.6 ± 1.34	3.55 ± 1.8

In 26 of 42 patients, a blood sample was obtained prior to KT to quantify pre-KT viremia (pTTV at T0). The presence of TTV DNA was detected in each sample collected, with a mean viral load of 5.50 copies/mL. In 7 of the 42 patients, a urine sample was also collected prior to KT: TTV DNA copies were detected in three samples.

Correlations between variables are reported in Supplementary Table S1. Regression analyses (age and sex as covariates) showed that pTTV levels at T0 were associated with dialysis years (for each increase in dialysis year, DNA copies increased by 0.21 ± 0.07 SE, p = 0.005) (Table 4.4).

Table 4.4. Association between baseline pTTV DNA copies (dependent variable) and baseline characteristics (generalized regressions for repeated measures, age and sex as covariates). SE: standard error.

Parameter	**N**	**Estimate**	**SE**	**p-Value**
Age	26	0.02	0.02	0.31
Sex (men)	26	-0.11	0.50	0.83
Dialysis years	26	0.21	0.07	0.005
Creatinine (mg/dL)	26	-0.05	0.10	0.62

4.1.1. TTV Kinetics after KT

We then analyzed the associations between TTV levels over time post-transplantation and their potential determinants. TTV DNA levels were measured at baseline and during follow-up (n = 363 measurements). Immediately after KT, the pTTV load (medium at day 15: 4.54 ± 1.77 log copies/mL) was lower than immediately before KT (medium: 5.26 ± 1.31 log copies/mL), with

a later increase after month 1 (Table 4.5). An association between time elapsed since KT and TTV emerged is as follows: both pTTV and uTTV increased 0.02 log copies/mL per day post-KT (SE = ±0.004, $p < 0.0001$; Table 4.6). The increase was evident after the first month (Figure 1a,b): +0.021 log copies/mL (SE = 0.005, $p < 0.0001$) and +0.029 log copies/mL (SE = 0.006, $p < 0.001$) per mL of plasma and urine, respectively (Herrmann, Sandmann et al. 2018).

A positive linear relationship between uTTV and pTTV was observed (Figure 1c): out of 301 observations, each 1 log copies/mL increase in uTTV corresponded to an increase in pTTV viral load of 0.56 log copies/mL (SE = 0.08, $p < 0.0001$). An increased TTV level was observed in men (plasma, 0.94 ± 0.48, $p = 0.04$; urine, 1.35 ± 0.48, $p = 0.005$). No association was found between plasma or urine TTV levels during follow-up and other patients' baseline characteristics, types of administered induction, maintenance immunosuppressive therapy, or circulating biomarkers, except for an inverse association between pTTV and plasma creatinine. It is noteworthy that a correlation was observed between pTTV and uTTV and CMV viremia ($p < 0.001$ and 0.050, respectively).

Table 4.5. Medium plasmatic creatinine, tacrolimus (FK), pTTV, and uTTV at selected time points.

Day	**Number of Measurements at the Selected Time**	**Creatinine (mg/dL)**	**FK (ng/mL)**	**p TTV (log copies/mL)**	**uTTV (log copies/mL)**
0	41	7.39 (±2.64)	-	5.26 (±1.31)	1.30 (±1.83)
15 (11-20)	46	4.38 (±3.04)	11.16 (±5.06)	4.54 (±1.77)	2.17 (±2.04)
25 (21-30)	33	3.009 (±2.48)	9.59 (±3.04)	4.33 (±2.13)	1.49 (±1.87)
35 (31-40)	25	2.17 (±1.44)	9.37 (±3.13)	4.40 (±2.49	1.70 (±2.31)
45 (41-50)	23	1.80 (±0.69)	9.61 (±2.99)	4.93 (±1.99)	1.85 (±1.96)
55 (51-60)	15	1.58 (±0.41)	8.81 (±3.22)	5.76 (±2.11)	2.78 (±2.37)
65 (61-70)	23	1.61 (±0.49)	9.77 (±3.16)	5.90 (±1.97)	3.09 (±2.11)

75 (71-80)	11	1.78 (±0.99)	8.78 (±1.65)	7.03 (±1.57)	4.45 (±2.08)
85 (81-90)	12	1.73 (±0.97)	10.58 (±5.67	5.67 (±3.13)	3.02 (±2.47)
95 (91-100)	11	1.62 (±0.36)	8.65 (±1.37)	6.14 (±1.24)	4.00 (±1.98)
>100	43	1.54 (±0.53)	9.02 (±2.17)	7.00 (±2.08)	4.64 (±2.04)

Table 4.6. Associations between plasma or urine TTV DNA copies, measured at baseline and follow-up, and clinical and laboratoristic parameters. Generalized regressions for repeated measures, age, and sex as covariates.

		Plasma TTV			**Urine TTV**	
Parameter	**N**	**Est ± SE**	**P-Value**	**N**	**Est ± SE**	**P-Value**
Age	346	0.02 ± 0.02	0.16	303	0.02 ± 0.01	0.06
Sex (men)	346	0.94 ± 0.48	0.04	303	1.35 ± 0.48	0.005
FK trough levels (ng/mL	305	0.04 ± 0.05	0.42	281	−0.02 ± 0.06	0.75
Leukocytic count (10^9/mL)	261	−0.07 ± 0.08	0.34	239	−0.06 ± 0.08	0.45
Lymphocitic count (10^9/mL)	258	0.23 ± 0.32	0.47	236	0.40 ± 0.26	0.13
Creatinine (mg/dL)	343	−0.15 ± 0.05	0.007	301	−0.18 ± 0.07	0.01
CMV viral load (log	275	0.15 ± 0.04	<0.0001	249	0.12 ± 0.06	0.053
Double induction therapy	346	0.62 ± 0.51	0.22	303	0.20 ± 0.52	0.70
Days elapsed since	346	0.02 ± 0.004	<0.0001	303	0.02 ± 0.004	<0.0001

uTTV	301	0.56 ± 0.08	<0.0001	-		
Days since KT *	346	0.02 ± 0.004	<0.0001	303	0.02 ± 0.004	<0.0001
uTTV *	301	0.56 ± 0.08	<0.0001	-		
CMV viral load (log) *	275	0.15 ± 0.03	<0.0001	227	0.12 ± 0.06	0.050

* Adjusted for number of years the patient underwent dialysis and type of induction therapy, single or double induction with rabbit anti-thymocyte globulin (rATG).

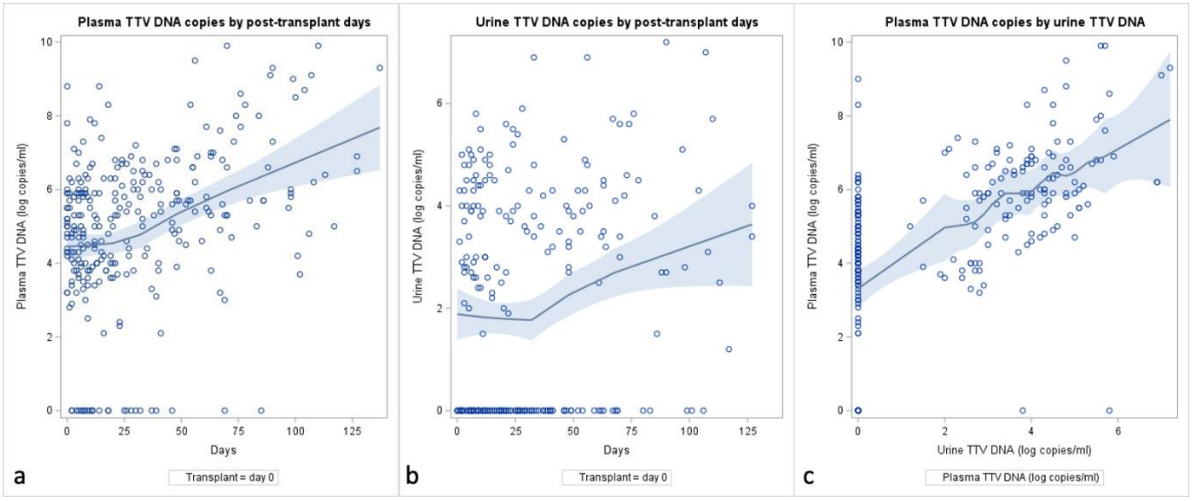


Figure 1. Changes in mean levels of plasma (panel a) and urine (panel b) TTV during follow-up. (Panel c): Change in pTTV by uTTV. Locally weighted regression (PROC SGPLOT with LOESS statement in SAS) using a scatterplot smoothing method that automatically determines the optimal smoothing parameter.

4.1.2. TTV Viral Load and Infection Events

All subjects were monitored for infectious complications during follow-up. To assess the association between TTV viral load and infection, we considered all episodes of infection, irrespective of the etiological agent or the severity of such episodes: both viral (including CMV infection or reactivation and VZV reactivation) and bacterial (with Klebsiella pneumoniae and Escherichia coli being the most commonly detected pathogens) infections, ranging from asymptomatic infections with positive cultures to severe infections such as urosepsis and enterocolitis.

We performed regression analyses to identify the association between events that occurred during follow-up (dependent variables) and TTV viral load (Table 4.7).

Table 4.7. Associations between plasma or urine TTV DNA copies, measured at baseline and follow-up, and events during follow-up. Generalized regressions, age, and sex as covariates

		Plasma TTV (Log Copies)			**Urine TTV (Log Copies)**			
	N	**Days**	**Est ± SE**	**p-value**	**N**	**Days**	**Est ± SE**	**p-value**
CMV	275	115	0.06 ± 0.01	<0.0001	249	105	0.06 ± 0.02	0.003
Infection (±1 day) *	346	160	0.04 ± 0.01	0.002	303	146	0.05 ± 0.01	0.0003
Bacterial infection	346	40	0.00 ± 0.01	0.84	303	38	0.02 ± 0.01	0.007
Viral infection	346	19	0.01 ± 0.01	0.13	303	17	0.01 ± 0.01	0.88
CMV reactivation	339	94	0.04 ± 0.0	0.012	296	86	0.04 ± 0.02	0.015

* Positive day: positivity on that day or on the previous and following day when unmeasured; days without measurements have been considered as negative days.

When all time points were considered, a higher pTTV was observed when clinical or laboratory signs of ongoing infection were present (mean: 5.735 log copies/mL; 95% CI: 5.392–6.079) compared to time points when no signs of infection could be detected (mean: 4.057 log copies/mL; 95% CI: 3.573–4.541; p value < 0.0001). Similarly, uTTV was significantly (p value = 0.0009) higher during ongoing infections than when no signs of infection were present (mean: 2.593 log copies/mL (95% CI: 2.143–3.042) versus 1.581 log copies/mL (95% CI: 1.169–1.993). The prevalence of infections increased across increasing quintiles of plasma and urine TTV levels (p < 0.0001 and p = 0.0005, respectively; Table 4.8). Interestingly, there were no differences in leukocyte or lymphocyte counts between the two groups (Figure 2a).

Table 4.8. Prevalence of infection days across quintiles of plasma and urine TTV DNA levels.

	pTTV			**uTTV**		
	No Inf	Infection	Total	No Inf	Infection	Total
Lowest quintile	51 71.8%	20 28.2%	71 20.5	82 66.1%	42 33.9	124 40.9%
2nd quintile	39 51.9%	27 40.9%	66 19.1	15 57.7%	11 42.3	26 8.6%

3rd quintile	42 62.7%	25 37.3%	67 19.4	32 61.5%	20 38.4	52 17.2%
4th quintile	45 59.2%	31 40.8%	76 22.0	21 41.2%	30 58.8	51 16.8%
Highest quintile	25 37.9%	41 62.1%	66 19.6	21 41.0%	29 58.0	50 16.5%
Total	202 58.4%	144 41.6%	346 100	171 61.9%	132 38.1	303 100%

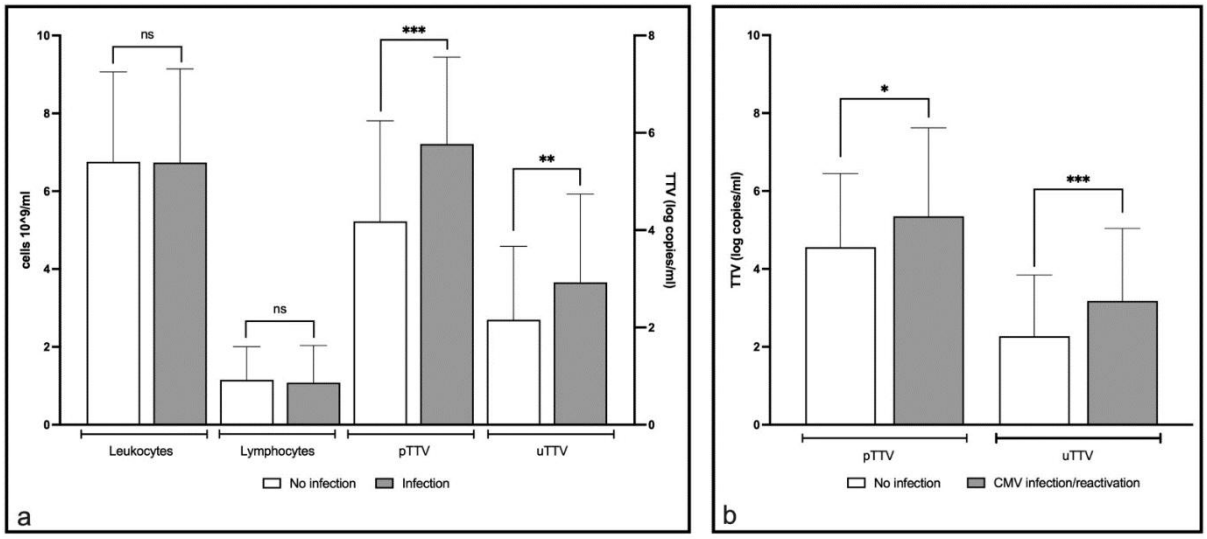
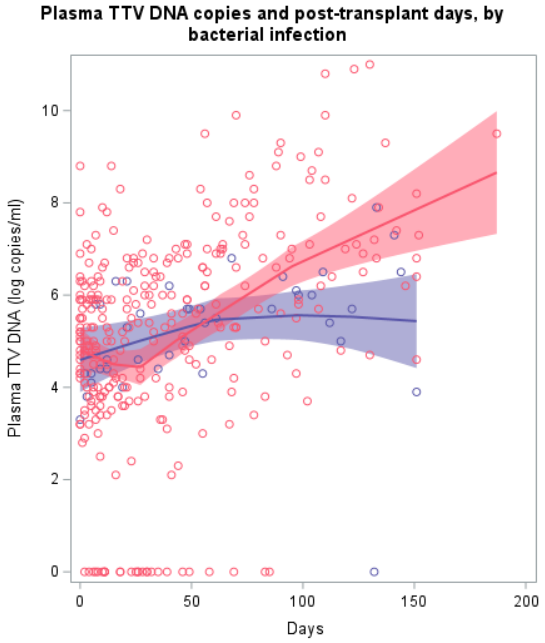
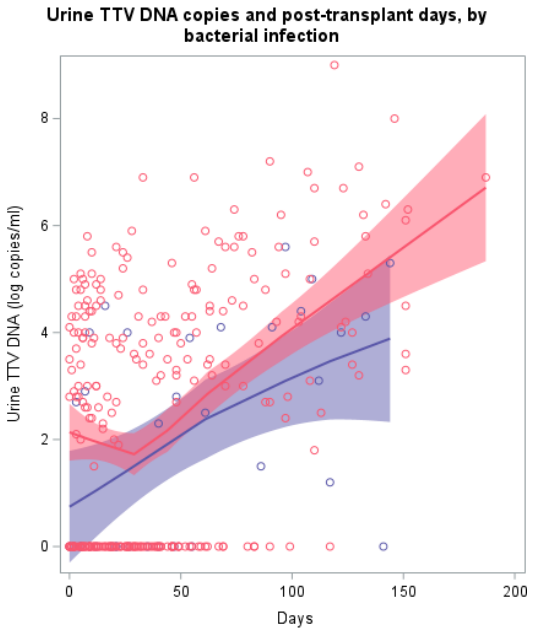


Figure 2. (a) differences between non-infection and infection events. (b) differences between non-infection and cases of CMV infection or reactivation (t test). ns: not significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0$.

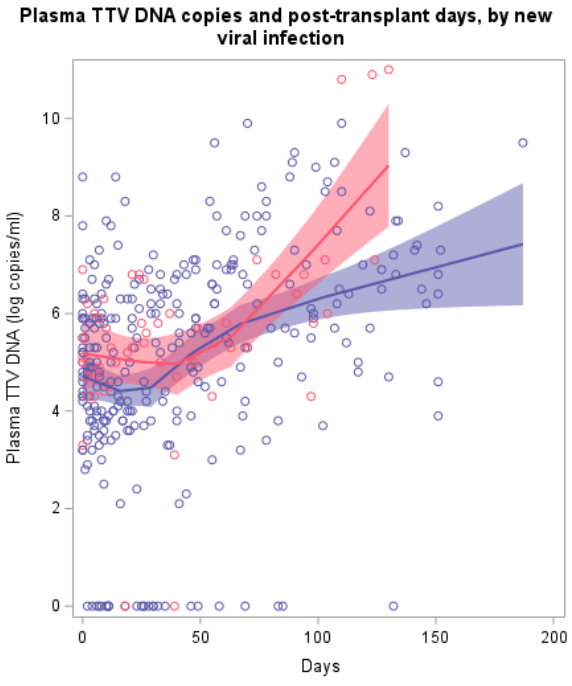
Six of 42 patients presented with negative CMV serostatum at transplantation, 4 of them (66.6%) developed CMV infection; three of four patients presented symptomatic CMV disease (66.66%), with a registered viral load of up to 241,782 copies/mL. CMV reactivation occurred in 26 patients (61.9%) and was treated with valganciclovir, except in a few cases where spontaneous regression was observed. In patients with CMV reactivation or primary infection, an increase in TTV viral load was observed that preceded or coincided with peak CMV viremia. Interestingly, a statistically significant association was found between plasma CMV viral load and uTTV viral load (Figure 2b). Figure 3 shows the distribution of pTTV or uTTV values according to days from transplantation, stratified for subjects who did or did not develop an infection during follow-up. The locally weighted regression shows a decreasing trend in the first 30 days, followed by an increase in plasma and urine TTV levels in the following months. The curves of both groups resulted to be similar and no statistically significant differences were

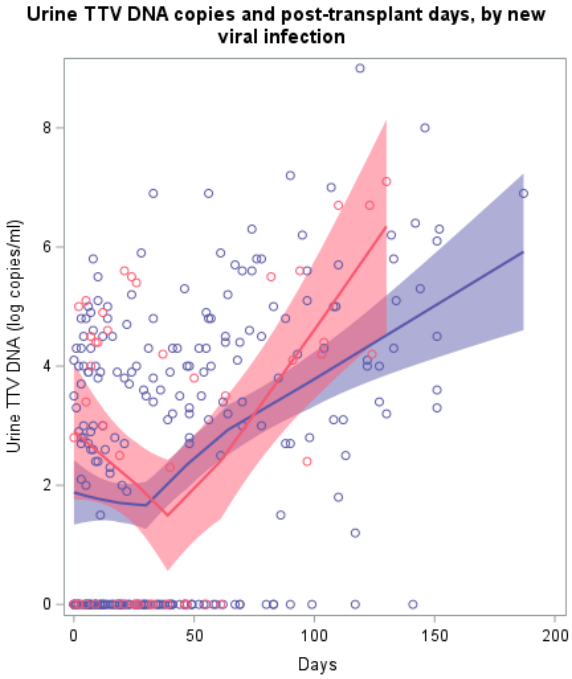
observed comparing plasma or urine TTV values of subjects with vs. without an infection during follow-up. This result did not change in multivariate analyses using, beyond age and sex, immunosuppression and days from transplant as covariates, and did not change restricting the analysis to the first month or to the following months. Similarly, in the Cox regression analyses using infection at the following time point as an event, no statistically significant associations were found between plasma or urine TTV levels and the event.





(a) **(b)**





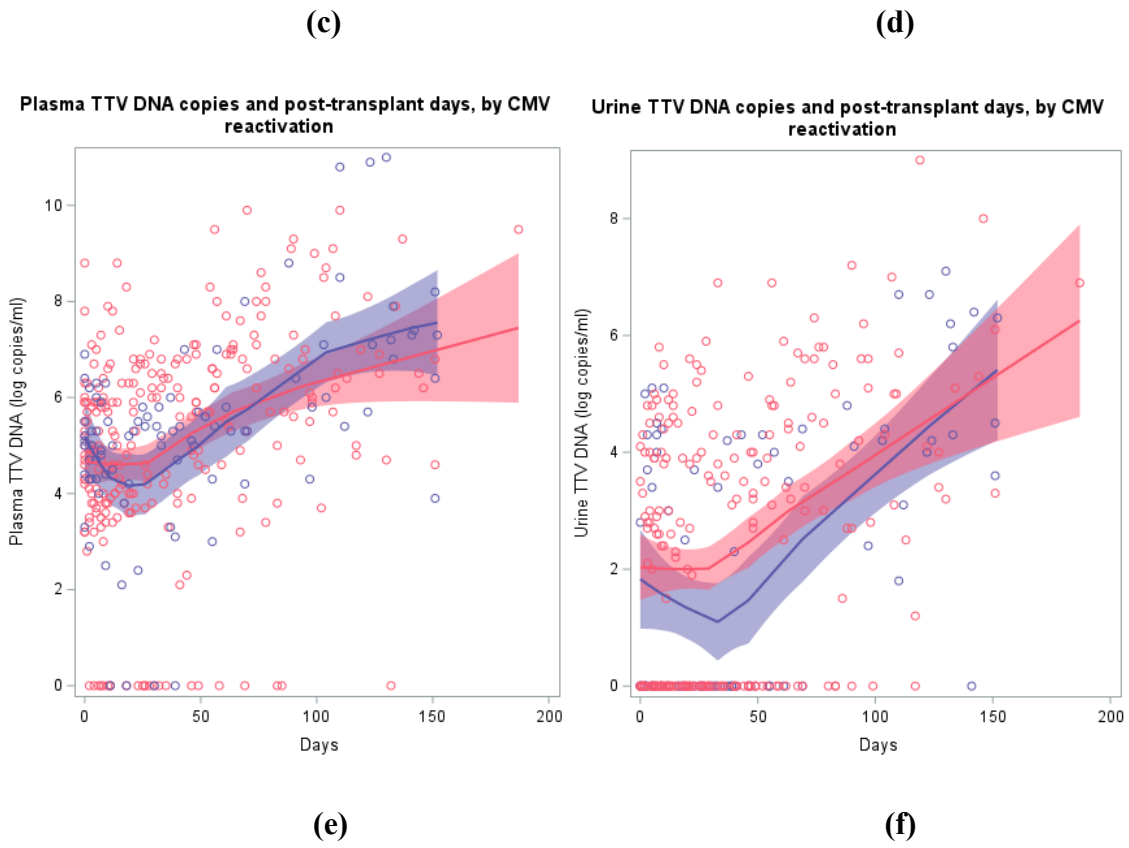
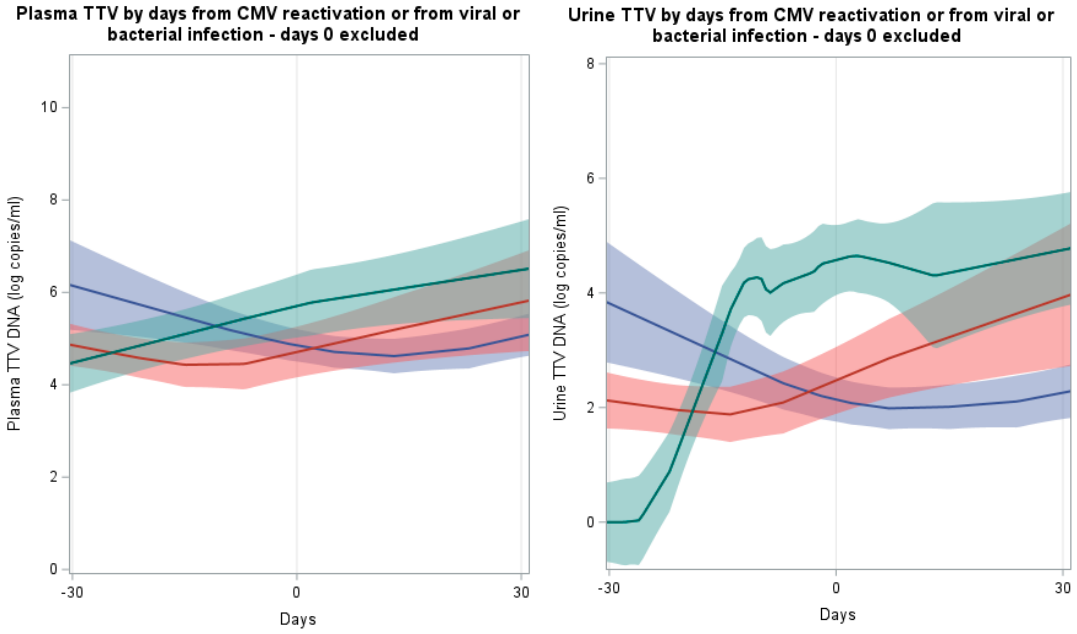


Figure 3. Distribution of plasma (left) or urine (right) TTV log copies by days from a bacterial (panel a,b) or viral (panel c,d) infection, or CMV reactivations (panel e,f) for subjects with (red) or without (blue) infections. Panel, plasma or urine, infection type and, for infection vs. no infections, total number of subjects, measurements and infection events: (a), plasma, bacterial, 35, 305, 75 vs. 7, 41, 0; (b), urine, bacterial, 35, 268, 66 vs. 7, 35, 0; (c), plasma, viral, 8, 54, 22 vs. 34, 292, 0; (d), urine, viral, 8, 47, 19 vs. 34, 256, 0; (e), plasma, CMV reactivations, 26, 255, 94 vs. 16, 92, 0; (f), urine, CMV reactivations, 26, 22, 86 vs. 16, 81, 0.

Figure 4 showed the distribution of plasma or urine TTV according to distance from the infection day, in the subgroup of subjects who developed an infection, to better focus on the time trend of TTV levels within subjects who developed a specific infection. The day of infection positivity was fixed at day 0 and the TTV curves of each subject, for the three infection types, were thus aligned and centered at their day 0. The locally weighted regression analyses showed that subjects with a new viral infection, in the days before the infection, reported an increase in both plasma and urine TTV viral load. An increase has been observed also in the case of CMV reactivation, starting 7 days before the event, while in the case of bacterial infection, an increase has been observed starting 15 days after the infection event. Although the increasing trends in the days before the infection suggest a potential use of TTV levels as predictors of new viral infection events or a CMV reactivation, the number of subjects was low to conduct an analysis of predictive abilities.



(a) (b)

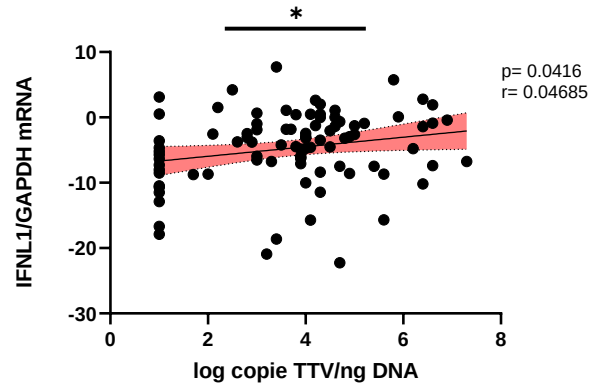
Figure 4. Plasma (panel a) or urine (panel b) TTV levels in subjects with a bacterial (blue) or viral (green) infection, or a CMV reactivation (red) during follow-up, according to distance from the day with the infection (day of positivity for infection has been fixed at 0 to align and center the curves); days 0 excluded. Total number of subjects, measurements, and infection events (panel a) plasma TTV levels, bacterial infections (35, 305, 75), viral infections (8, 54, 22), and CMV reactivations (26, 255, 94); (panel b) urine TTV levels, bacterial infections (35, 268, 66), viral infections (18, 47, 19), and CMV reactivations (26, 222, 86).

4.2. Correlation between cytokines expression analysis and TTV viral load

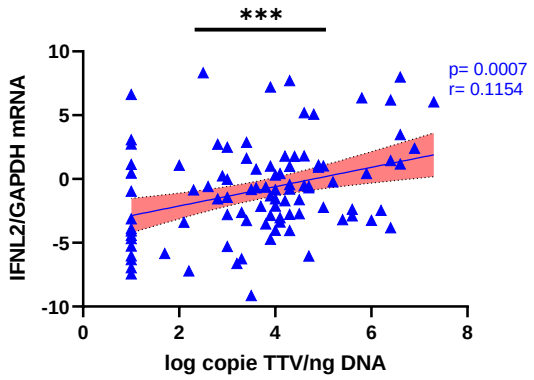
Genes (IFNL1, IFNL2, IFNB1, IL1B and IL6) expression was assessed with respect to the housekeeping gene GAPDH. Quantitative results of cytokine expression levels were calculated by using qRT-PCR according to manufacturer instructions.

The mRNA expression of all cytokines was investigated in relation to TTV log copies/ng DNA values across 97 TTV-positive samples. This study analyzed the distribution of interferon levels in relation to viral load. Among the members of the IFN-III family, both IFNL1 and IFNL2 exhibited a positive correlation with viral load, as depicted in Figure 4.2.1 (panels 1 and 2). The expression of IFNL1, as indicated by a Pearson correlation coefficient, showed a significant correlation with p-value 0.0416. This suggests that IFNL1 levels vary meaningfully with viral load in this cohort. Additionally, a strong correlation was observed for IFNL2. The mRNA expression of IFNL2 demonstrated a significant correlation with viral load in TTV-positive samples by a Pearson correlation coefficient with a p-value of 0.0007, suggesting a robust association with TTV viral load.

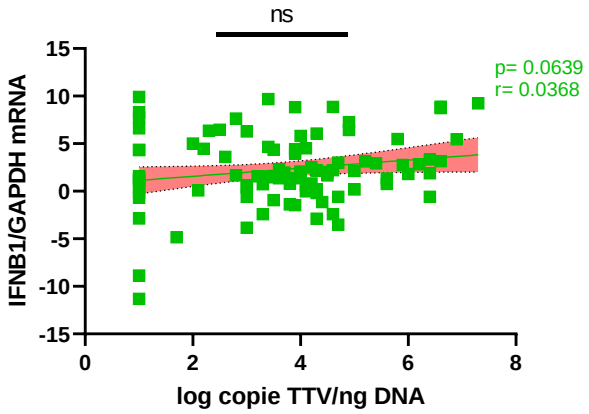
In contrast, in figure 4.2.1 (panel 3,4 and 5), the transcript levels of the pro-inflammatory cytokines IFN-B1, IL-1B and IL-6 exhibited non-significant correlation. Overall, findings indicate that higher viral loads are associated with increased levels of specific interferons and pro-inflammatory cytokines, suggesting a potential interplay between viral replication and the immune response.



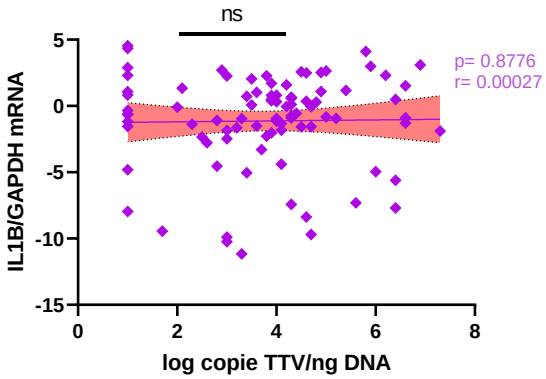
(a)



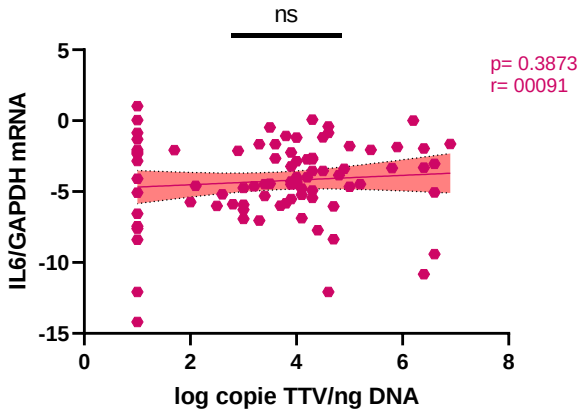
(b)



(c)



(d)



(e)

Figure 4.2.1: (a-e) IFNL1 (a), IFNL2 (b), IFNB1 (c), IL1B (d), and IL6 (e) mRNA expression is plotted against TTV log copies/ng DNA in 96 TTV-positive patients. Each dot represents a patient. Linear regression lines (continuous line) and 95% confidence interval (dotted line and shaded area) are depicted in red. Pearson correlation coefficients (r) and p value (p) are indicated. ns, not significant ($p > 0.05$); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

4.2.1. TTV DNA viral load in the patient age groups

To investigate whether a general loss of the immune system's ability to respond to viruses in elderly persons is mirrored by TTV, this study assessed whether the TTV DNA load, which is supposed to reflect the extent of the immune response to TTV, is associated with a difference in age. Total 97 positive samples were categorised into three major age groups as young age group (1-18 years), middle-age group (20-63 years) and elderly group (65-92 years). The mean TTV DNA values of the TTV-positive samples were 4.183 log copies/ng in the young group, 3.200 log copies/ng in the middle-aged group, and 3.500 log copies/ng in the elderly group. A post hoc Tukey's HSD test was performed and as shown in Fig. 1, revealed that there was a significant association between age groups and TTV viral load (p= 0.0458).

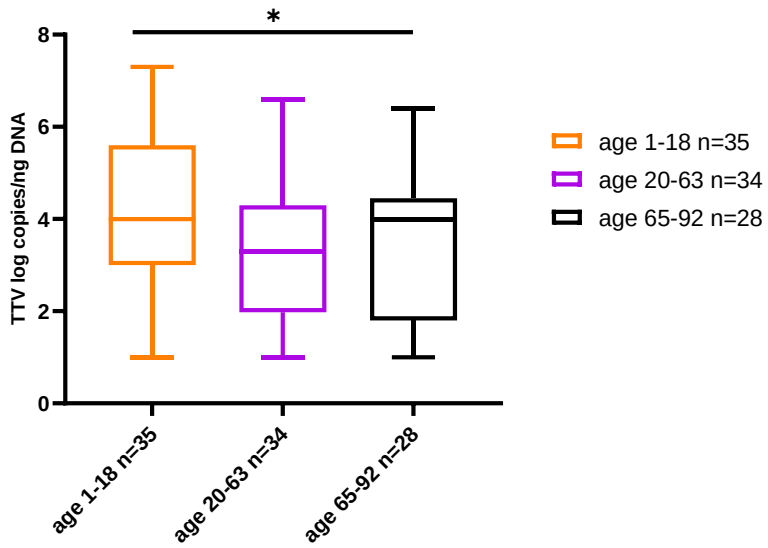


Figure 4.2.2: TTV DNA log copies/ng between three age groups. The ANOVA post hoc Tukey's HSD test was performed to compare mean values between the age groups. Nonsignificant p values are shown as ns. Thick horizontal bars in the box represent the median value of each group.

4.2.2. TTV viral load correlation with cytokines in the patient age groups

The results of TTV on interferons and interleukins vary across different age groups. This study then evaluated how IFN gene expression relates to the age of patients fall in the category of 1-18, 20-63 and 65-92 years old.

Figure 4.2.3 (a-e) presents the correlation analysis between TTV and various cytokines across different age categories. In individuals aged 1 to 18, figure 4.2.3 (panel a), the analysis reveals no significant correlation between TTV and the cytokines IFN1, IFN2, IFN-β1, IL-1β, and IL-6. Conversely, among individuals aged 20 to 63, a significant positive correlation is observed between TTV and IFN2, with a p-value of 0.0255, indicating that higher levels of TTV are associated with increased IFN2 levels in this age group. However, for the oldest age category (65 to 92), the results indicate that there is no significant correlation between TTV and the assessed cytokines. These findings highlight the variability of immune responses to TTV across different age groups, suggesting that age may play a crucial role in the relationship between TTV and cytokine expression. Overall, this table emphasizes the importance of considering age as a factor when studying the immunological aspects of TTV.

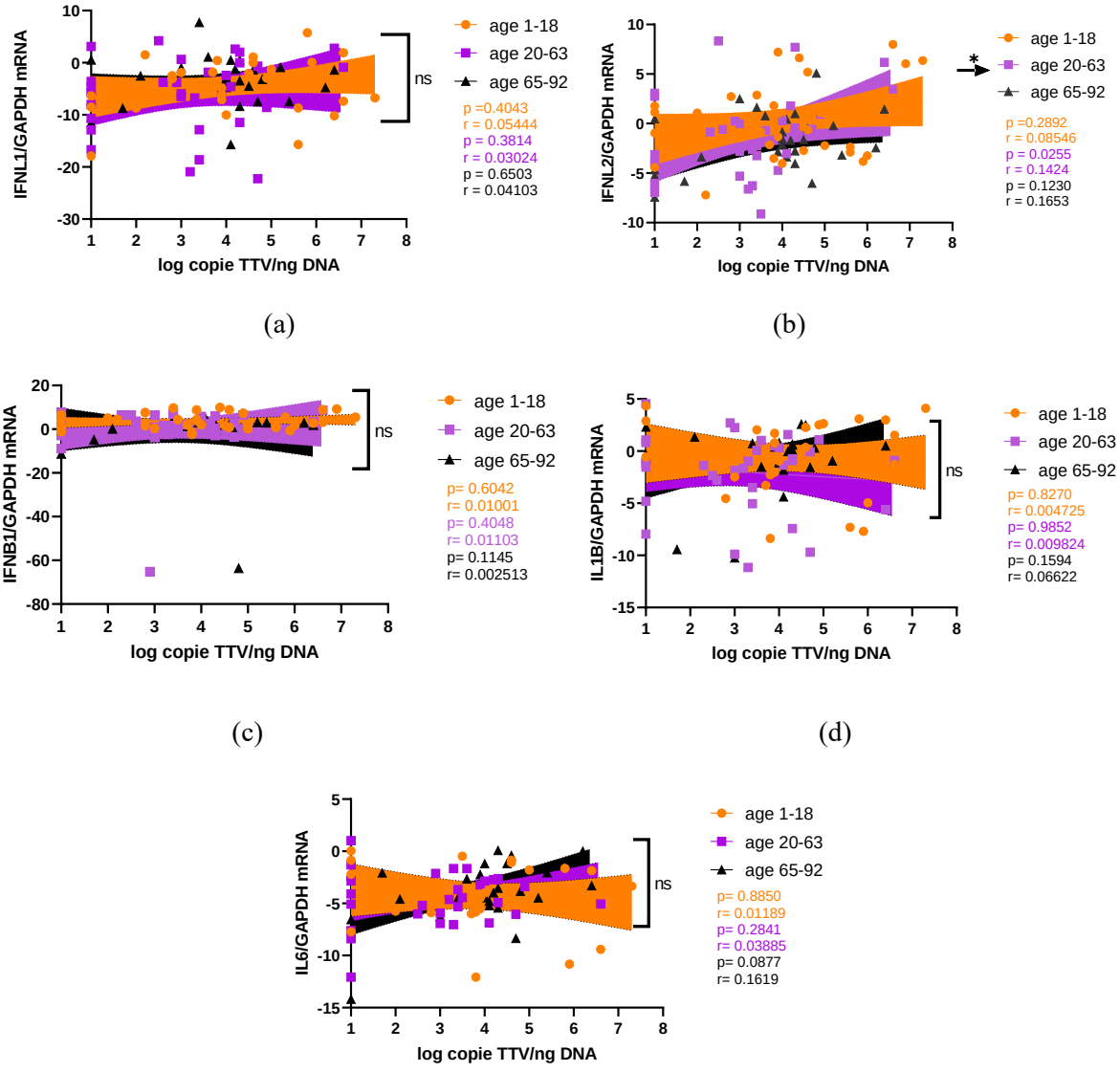


Figure 4.2.3: (a-e) IFNL1 (a), IFNL2 (b), IFNB1 (c), IL1B (d), and IL6 (e) mRNA expression is plotted against log copies TTV/ng DNA in 97 TTV-positive patients were categorised into three major age groups as young age group (1-18 years, 35, orange dots and lines), middle-age group (20-63 years, 33, purple dots and lines) and elderly group (65-92 years, 29. Black dots and lines). Each dot represents a patient. Linear regression (continuous lines) and 95% confidence interval (dashed line and shaded area) are depicted. Spearman correlation coefficients (r) and p value (p) are indicated in orange, purple and black for young, middle age and elderly age patients, respectively. ns, not significant ($p > 0.05$); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

5. Discussion

TTV has been proposed as a marker to assess the immune status of SOT recipients, and it is believed that it may be useful to modulate the immunosuppressive load and prevent complications due to insufficient or excessive immunosuppression, such as rejection and opportunistic infections. Early identification of inadequate immunosuppression could create an opportunity to enhance immunosuppressive drug therapy. This tailored adjustment of immunosuppression may help minimize alloreactivity and subsequent graft damage, presenting a promising strategy that could be evaluated in a randomized controlled trial (Doberer, Schiemann et al. 2020, Doberer, Haupenthal et al. 2021).

It is known that patients with a compromised immune system, such as KT recipients, have an increased TTV plasma load. Several studies have found a positive association between TTV load and risk of infection and a negative association between TTV load and risk of rejection (Strassl, Schiemann et al. 2018, Doberer, Haupenthal et al. 2021, Grenda 2021, Zeng, Tang et al. 2023). Most of these studies focus on the role of plasma TTV load, but not on the role of urinary TTV load.

In recruited patients, in the immediate pre-transplant period, the mean pTTV viral load was 5.30 log copies per mL plasma, while only three patients provided a pretransplant urine sample, and there was no evidence of urinary excretion of TTV in any subjects with residual diuresis. A decrease in pTTV was observed immediately after KT. A decrease in viremia in the immediate post-transplant period is also described in previously published studies and appears to be justified by the site of viral replication, consisting of certain subpopulations of peripheral blood T lymphocytes, and by the lymphopenicising effect of induction therapy (Focosi, Macera et al. 2015, Herrmann, Sandmann et al. 2018, Doberer, Schiemann et al. 2020). In contrast to other studies published in the literature, which described an earlier increase in viral titers around day 20 post-KT (Doberer, Haupenthal et al. 2021, Zeng, Tang et al. 2023), in this study, an increase in pTTV was observed from day 30 post-KT.

In the first hypothesis, these differences can be attributed to the cohort of patients considered; in fact, most studies describing TTV kinetics published to date have recruited either a pediatric population or lung or combined kidney–pancreas transplant recipients, in whom the kinetics of TTV are different due to differences in the immune system and immunosuppressive regimen. In the second analysis, there are differences related to the immunosuppressive therapy administered, as more than 10% of the recruited subjects were receiving their second or third KT, and several patients had a high number of panel reactive antibodies (PRA), necessitating

the use of dual induction therapy with basiliximab and thymoglobulin, which is known to be more lymphopenic than basiliximab alone.

There is a paucity of data in the literature regarding the role of urinary TTV load which hampers our understanding of its clinical relevance. Our data demonstrated that the viral load of uTTV follows similar kinetics to that of pTTV, with an initial decrease and an increase at the end of the first month. This suggests that uTTV dynamics might reflect systemic changes in TTV infection, relevant for monitoring immune responses in patients. The early decline may indicate an immune response or effective clearance, while the subsequent rise could reflect viral resurgence or immune shifts. Further research is essential to clarify the implications of urinary TTV load in clinical practice and its potential role in immune modulation.

Similar trends in pTTV and uTTV kinetics are justified by the existence of a positive linear relationship between pTTV and uTTV: each increase of 1 log copies/mL in uTTV corresponds to an increase of 0.55 log copies/mL in pTTV viral load (p-value < 0.0001). No study published to date has described the kinetics of the urinary viral load of TTV in KT recipients, so comparisons could not be made. The increase in viral load after transplantation seems to reflect the increasing degree of immunosuppression after transplantation until stabilization occurs towards the end of the third month. This appears to be in line with the current consensus that the first three months after transplantation are the most critical period, when there are greater fluctuations in the concentration of immunosuppressive drugs, resulting in a greater risk of complications.

Because of its correlation with immune system function, Torque Teno Virus has been proposed as a prospective marker to predict infection and rejection in SOT recipients. All subjects enrolled in this study were followed to assess any episodes of rejection and infectious complications that occurred during follow-up. It was not possible to assess the endpoint related to rejection, as no biopsy-confirmed episodes of rejection occurred in any of the subjects enrolled in the study during the entire follow-up period. For each time point, weekly, or even more frequently in the immediate post-transplant period, we evaluated the presence of clinical and laboratory signs suggestive of infection, regardless of clinical manifestation or etiological agent, and observed that pTTV load was 40 times higher (p value < 0.0001) when infection was ongoing compared to time points when the patient did not manifest signs of infection. Similarly, there was a statistically significant association (p < 0.0001) between urinary TTV

load and the presence of infection, with a 10-fold higher urinary TTV load in ongoing infections.

Indeed, there are known associations between TTV levels and infectious events; Doberer and coworkers (Doberer, Haupenthal et al. 2021) report that each increase of 1 log copies/mL corresponds to an 11 percent increase in infectious risk ($p < 0.001$), while Fernández-Ruiz and coworkers highlight that there is already increased risk of developing opportunistic infections at a viral titer of 3.15 log copies/mL (Fernández-Ruiz, Albert et al. 2020). A meta-analysis by van Rijn and coworkers (Van Rijn, Roos et al. 2023) reports how different studies identify different cut-offs at different time points after transplantation, making it necessary to identify critical values at predetermined time points or to create a model that can be applied regardless of the time elapsed since transplantation to assess infectious risk.

Since we observed no differences considering distance from transplantation, while we found a changing trend in days before viral infections, we suggest considering changes over time in the same subjects, irrespective of time distance from transplantation. Our study suggests performing combined measurements of plasma and urine TTV levels, to potentially predict new viral infections or CMV reactivations, one of the major infectious agents causing morbidity and mortality in transplant recipients (Anderson-Smits, Baker et al. 2020). However, large studies measuring both plasma and urine TTV levels, along with FK, at frequent time points and for a long follow-up should be conducted to study predictive abilities of TTV levels.

The study on cytokine profiling showed a significant positive correlation between TTV and the expression levels of key cytokines such as IFNL1 ($p = 0.0416$) and IFNL2 ($p = 0.0007$). Nevertheless the nature of this interaction is at the moment unknown, this upregulation could contribute to the body defence against other pathogens by priming the immune system.

6. Conclusions

The number of studies delineating the role of TTV as a marker of immunosuppression is increasing. There is evidence that TTV is useful not only in estimating the level of immunosuppression, but also in predicting the occurrence of potential complications. This is because TTV seems to reflect overall immune function.

While plasma TTV levels at T0 were associated with dialysis years, no association was found between plasma and urine TTV levels during follow-up or in other patients' baseline characteristics, type of administered induction, or maintenance immunosuppressive therapy, or circulating biomarkers, except for an inverse association between pTTV and plasma creatinine, lymphocytic count, and leukocytes. It is noteworthy that an elevated TTV level was observed in males.

The study presents significant findings regarding the relationship between plasma TTV (pTTV) and urinary TTV (uTTV) levels, emphasizing their role as markers of immune status in patients, particularly those who have undergone transplantation. It establishes a positive linear relationship between pTTV and uTTV, indicating that increases in one correlate with increases in the other. This consistency suggests that both plasma and urine samples can provide a reliable measure of viral presence in the body, offering a comprehensive understanding of the patient's immune response.

Quantification of TTV in both plasma and urine proves to be crucial for assessing immune status. This dual approach allows healthcare providers to gain deeper insights into how well the immune system is functioning, especially in immunocompromised patients. By monitoring TTV levels, clinicians can better tailor immunosuppressive therapies to individual patient needs. This personalized approach not only aims to minimize the risk of infections but also reduces the potential side effects associated with over-immunosuppression.

Furthermore, the study highlights the predictive value of TTV quantification for infectious complications. By serving as an early warning system, TTV levels can help identify patients at risk of developing infections, which is critical in managing the health of vulnerable populations, such as transplant recipients. Among the notable findings is the strong correlation between urinary TTV levels and cytomegalovirus (CMV) infection or reactivation. This relationship suggests that monitoring urinary TTV could be a valuable strategy for tracking CMV activity, which is particularly important given the severe complications that CMV infections can pose in these patients.

The study also addresses the limitations of traditional monitoring methods, such as leukocyte or lymphocyte counts, which may not reliably reflect immune status in transplant patients. This reinforces the need for alternative strategies, like monitoring TTV levels, to provide a more accurate picture of a patient's immune function. Interestingly, the research found no significant differences in TTV levels based on the time elapsed since transplantation. However, it did observe changes in TTV levels in the days leading up to viral infections, suggesting that continuous monitoring over time is essential. This approach allows for timely interventions that can significantly enhance patient outcomes.

In conclusion, the findings of this study support the use of urinary TTV as a non-invasive and reliable marker for monitoring immune status and predicting infections, particularly CMV reactivation, in transplant patients. By adopting this method, healthcare providers may improve the management of immunosuppressive therapies, ultimately leading to better patient care and outcomes through proactive and personalized treatment strategies.

Further studies in larger populations are needed to assess the timing of pre-infection TTV elevation and to evaluate predictive metrics, as well as the identification of predetermined time points, cut-offs, and the development of validated and easily applicable models.

This study also shows that IFNL1 and IFNL2 are significantly correlated with TTV viral load, while other pro-inflammatory cytokines are not significantly correlated. The complexity of the interaction between TTV and the immune system requires further research to elucidate the mechanisms involved. Future studies should explore the functional implications of these findings and their potential relevance in the clinical setting. The variability of cytokine expression and TTV correlation across age groups emphasises the need for further research to explore the immunological implications of TTV infection in different populations.

7. Future Directions

1. Further studies in larger populations are needed to evaluate the analytical metrics, as well as the identification of predetermined time points, cut-offs, and the development of validated and easily applicable models. Comprehensive large-scale studies should be conducted to investigate the predictive abilities of TTV levels by measuring both plasma and urine concentrations at regular intervals over an extended follow-up period. These studies would involve collecting samples from a diverse cohort of participants to ensure robust data, allowing researchers to establish a clear correlation between TTV levels and relevant clinical outcomes. By frequently assessing TTV levels alongside the immunosuppressive drug, tacrolimus (FK), researchers can gain insights into how fluctuations in these viral levels might correlate with patient responses and potential complications. The long-term follow-up would be essential in determining whether TTV levels serve as reliable biomarkers for immunosuppression effectiveness or for predicting the risk of graft rejection, thereby enhancing patient management strategies and outcomes in transplant settings. This approach could ultimately lead to more personalized treatment plans and improved monitoring protocols for patients undergoing immunosuppressive therapy.

2. Long-term studies are essential to fully understand the intricate relationship between Torque Teno Virus (TTV) infection and immune modulation, particularly regarding its potential direct influence on cytokine production. Cytokines are critical signaling molecules in the immune system that regulate various immune responses, and any alteration in their production can have significant implications for immune function and overall health. By following patients over an extended period, researchers can gather valuable data on the dynamics of TTV infection and its correlation with changes in cytokine levels.

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