

# Clinical relevance of plasma virome dynamics in liver transplant recipients



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## ABSTRACT

**Background:** The role of the microbiome in liver transplantation (LT) outcome has received a growing interest in the past decades. In contrast to bacteria, the role of endogenous viral communities, known as the virome, is poorly described. Here, we applied a viral metagenomic approach to study the dynamic evolution of circulating viruses in the plasma of LT recipients and its effect on the clinical course of patients.

**Methods:** Patients chronically infected with hepatitis B virus (HBV) that received a LT due to endstage liver disease were included in this study. Longitudinal plasma samples were collected pre- and post-LT. Intact viral particles were isolated and sequenced on an Illumina HiSeq 2500 platform. Short read libraries were analysed with an in-house bioinformatics pipeline. Key endpoints were the dynamics of viral families and post-LT complications.

**Findings:** The initiation of immunosuppression induced a bloom of the *Anelloviridae* that dominated the post-LT plasma virome. A variety of post-LT complications were observed. Nephrotoxicity was reported in 38% of the patients and was associated with a high abundance of anelloviruses. Besides nephrotoxicity, 16 (67%) patients experienced flares of viral or bacterial infections in post-transplant follow-up. These flares were recognized by an increased burden of anelloviruses ( $p < 0.05$ ). Interestingly, no mortality was observed in patients infected with human pegivirus.

**Interpretation:** These findings suggest a diagnostic potential for the *Anelloviridae* family in post-LT complications. Furthermore, the impact of human pegivirus infection on post-transplant survival should be further investigated.

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## 1. Introduction

In patients receiving liver transplantation (LT), organ rejection, post-transplant infections and complications related to long-term immunosuppression such as nephrotoxicity and *de novo* malignancies are the main substantial threats [1]. All these complications are potentially related to viral infections or viral reactivations. Consequently, viral infections impose an adverse impact on the survival rate after LT [2]. For instance, cytomegalovirus (CMV) is the most common infectious pathogen that leads to graft failure in liver transplant recipients [3]. Therefore, liver transplanted patients are regularly monitored for CMV viremia and subjected to preventive

antiviral therapy in case of high-risk serological constellations [4,5]. Notably, patients diagnosed with viral hepatitis-related hepatocellular carcinoma (HCC) have a higher risk of adverse post-transplant outcomes compared to the non-viral HCC patients because of possible recurrent infections [6].

Over the past decades, there has been a growing interest in the role of endogenous microbes for the outcome of organ transplantation. Studies of the gut microbiota revealed that dysbiosis following liver transplantation is associated with impaired graft survival [7]. On a functional level, increased intestinal permeability, decreased abundance of beneficial commensal bacteria and an increase in pathogenic species may contribute to post-transplant infections and acute rejection [8]. However, there is still a large gap of information concerning the role of the virome in liver transplantation.

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## Research in context

### Evidence before this study

Immunosuppressive therapies have significantly improved the survival of organ transplant patients. However, these therapies also increase the chance of post-transplant complications including secondary infections and de novo malignancies. Mounting evidence suggest an important role of the microbiome in organ transplant survival and patient longevity. The viral part of the microbiome, called the virome, has been associated with the functioning of the immune system. The crosstalk between the virome and immune system could have important implications in organ transplantation medicine. However, limited information is available concerning the peculiar role of these 'commensal' viruses.

### Added value of this study

Patients that received a liver transplantation due chronic HBV infection were included in the study.

Viral metagenomic analyses of longitudinal plasma samples collected before and after liver transplantation revealed the presence of a divergent virus community. The initiation of immunosuppressive therapy induced a bloom of anelloviruses in both absolute abundance and species diversity. A variety of post-transplant complications were observed, including renal failure and infections. Remarkably, patients diagnosed with nephrotoxicity and post-transplant infections had a higher abundance of the *Anelloviridae* family in the blood samples. Both multi- and univariate analyses implied that the anellovirus abundance could be a proxy for immunosuppression and the possible risk of post-transplant complications. Besides the *Anelloviridae*, also human pegivirus (*Flaviviridae*) was observed. Previous studies have subscribed immunomodulatory effects to this virus in different clinical backgrounds. In our study, no mortality was observed in patients that were found positive for pegivirus. These findings clearly demonstrate the potential clinical relevance of the virome in post-liver transplantation complications.

### Implications of all the available evidence

The virome remains an understudied component of the human microbiome. This study continued to further explore the implications of viruses in human health. Based on our findings, specific members of the human blood virome could be used as a diagnostic marker for post-transplant complications. Little is known about the 'commensal' viruses that inhabit the blood stream and their interactions with the immune system. However, based on previous findings and the data presented in this study, these viruses could be attractive for developing innovative biomarkers and support clinicians in developing personalized treatment strategies.

The virome is the most abundant and genetically diverse fraction of the human microbiome [9]. The virome interacts with host genetic factors and other microbiome components to shape the immunophenotype [9,10]. This phenotype can affect the clinical outcome of infections with pathogens, inflammatory illnesses and interventions like organ transplantation [10]. Viral metagenomic studies in organ transplant settings demonstrated that some plasma virome components take advantage of the reduced immunocompetence imposed by immunosuppressive agents [4]. These findings suggest that the viral abundance of particular viruses could be a marker of immunocompetence in immunosuppressed patients. Besides the potential role as a prognostic biomarker of adverse events, virome components are also

related to positive outcomes in specific clinical manifestations. For example, the co-infection of human pegivirus (HPgV) with HIV has a beneficial impact on the survival of HIV infected individuals [11].

We hypothesized that the plasma virome and its dynamic evolution after transplantation might be associated with the clinical course of liver transplanted patients. To fill the current gap, we evaluated the impact of LT on the virome composition and dynamics before and after transplantation in a cohort of hepatitis B virus (HBV) infected individuals undergoing liver transplantation. HBV-infected patients provide a unique opportunity to study such virome-host interactions. They typically cure their HBV infection due to the liver transplantation that is accompanied by antiviral therapy and HBV hyperimmunoglobulin (HBIG) administration. However, these patients have a higher risk of hepatic malignancies, while the risk for liver transplant-related events (rejection, cirrhosis, re-transplantation) and death after transplantation are similar to non-HBV exposed recipients [12]. By analysing longitudinal plasma samples via next-generation sequencing and corresponding clinical data, we provide a comprehensive analysis of the dynamic evolution of the circulating virome and its associations with the clinical course in LT patients.

## 2. Methods

### 2.1. Study population and ethics statement

HBV infected patients diagnosed with end-stage liver diseases who received a liver transplantation at UZ Leuven (Belgium) between 2007 and 2014 were consecutively enrolled in this study. These patients are part of the chronic HBV cohort that is followed in UZ Leuven. Indications for liver transplantation other than HBV infection and a history of previous organ transplantations were used as exclusion criteria. This study was performed in accordance with the Declaration of Helsinki and was approved by the Ethics committee research UZ/KU Leuven, Belgium. All participants gave informed consent and were able to withdraw from the study at any moment.

Serial plasma samples were collected from the HBV biobank. In total, six longitudinal samples were collected per patient, two plasma samples before (samples A and B) and four (samples C, D, E and F) after liver transplantation. The two samples (A and B) before transplant were collected at  $41 \pm 45$  (A) and  $2 \pm 1$  (B) months pre-transplant, while four samples (C, D, E and F) after transplant were taken at  $0.5 \pm 0.2$  (C),  $5 \pm 1$  (D),  $13 \pm 6$  (E), and  $23 \pm 11$  (F) months post-transplant. Biochemical, serological, and virologic markers were assayed by the hospital laboratories. Moreover, demographic data including age, sex, country of origin, and medication were obtained from the patient clinical record.

### 2.2. Post-liver transplant treatment protocol

All patients received immunosuppressive therapy that consisted of tacrolimus (e.g.  $2 \times 1$  mg/d), mycophenolate mofetil (e.g.  $2 \times 250$  mg/d) and methylprednisolone. Dosages of immunosuppressive therapies were adapted according to the therapeutic window and clinical signs of side-effects. Initially, methylprednisolone was prescribed in high dose of  $2 \times 20$  mg/d and tapered in the following months until discontinuation after three months, depending on clinical conditions of the patients. Infection prophylaxis included a regimen of antibiotic and antiviral compounds. Prophylactic therapy with Valganciclovir (450 mg/d) was prescribed in case of donor and recipient CMV mismatch. Since all patients received a liver transplantation due HBV related end-stage liver disease, HBV prophylaxis was prescribed post-liver transplantation. Anti-HBV therapy consisted of intravenous injection of HBV antibodies and HBV nucleoside analogues.

### 2.3. Plasma sample processing and viral nucleic acid extraction

Plasma samples were treated with an optimized viral enrichment protocol. First, samples were sequentially filtered with 5.0  $\mu\text{m}$  polypropylene (Millipore) and 0.8  $\mu\text{m}$  polyethersulphone (Sartorius) filters. To reduce free-floating nucleic acids, filtrated plasma samples were treated with a cocktail of micrococcal nuclease (New England Biolabs) and benzonase (Millipore) and subsequently extracted with the QIAmp Viral RNA Mini Kit (Qiagen) without carrier RNA. To increase the yield of nucleic acids content, a random amplification step was performed using the Whole Transcriptome Amplification Kit 2 (WTA2, Sigma Aldrich) with an initial denaturation step at 95 °C instead of 70 °C. The extracted nucleic acids were subjected to 20 amplification cycles and purified using the MSB SPIN PCRAPACE kit (Stratec).

### 2.4. Sequencing library preparation and sequencing

NGS library preparation was performed using the Nextera XT DNA Library Preparation kit (Illumina) with a decreased tagmentation time to 4 min (input DNA of 1.2 ng/  $\mu\text{l}$ ) and halved reagent quantities to increase average DNA fragment sizes. The average fragment sizes of DNA libraries were evaluated using the High Sensitivity DNA Kit (Agilent) on a Bioanalyzer 2100 (Agilent). Libraries were quantified with the KAPA Library Quantification kit (Kapa Biosystems). Finally, sequencing was performed on a HiSeq 2500 platform (Illumina) for  $2 \times 150$  cycles, with a total of 10 million paired end reads per sample.

### 2.5. Bioinformatic analysis

NGS reads were analysed with an in-house bioinformatics pipeline. Raw reads were trimmed with Trimmomatic [13]. To remove non-viral sequences, reads were mapped against the human genome and a non-redundant database of contaminating sequences with Bowtie2 [14]. The remaining reads were *de novo* assembled with metaSPAdes and the assembled contigs were annotated by DIAMOND with the sensitive option using the Genbank's non-redundant database [15,16]. Moreover, an additional annotation was performed using BLASTn against the nucleotide reference database (NCBI database of January 2018). Bacteriophages were annotated using both MetaPhinder2 and VirSorter in 'virome decontamination' mode [17,18]. The assembled contigs were clustered to generate genome bins with 95% nucleotide identity and a minimal coverage of 80% using nucmer from the MUMmer package [19]. Finally, reads were mapped back against the clustered genomes with BMap and genome bins that mapped less than 100 reads were discarded from further analysis [20].

### 2.6. Anellovirus quantification

qPCR was carried out on 1:10 diluted extracted DNA/RNA samples. Universal forward (ACWKMGCAATGGCTGAGTTT) and reverse (CCCKWGCCCCGARTTGCCCT) primers were used targeting the UTR of the *Anelloviridae*. The qPCR-assay was developed for detecting and quantifying anelloviruses with a SYBR-green approach. Oligonucleotides (Eurogentec, Belgium) with known concentration were used for the standard curve to determine the sample concentrations. Per sample, the master mix consisted of 10  $\mu\text{l}$  qPCR BIO SyGreen Blue Mix Lo-ROX (Sopachem, Belgium), 2.5  $\mu\text{l}$  of both primers (10  $\mu\text{M}$ ), 4  $\mu\text{l}$  dNTPs, and 1  $\mu\text{l}$  H<sub>2</sub>O. Samples were analysed on a 7500 Fast real-time PCR system (Applied Biosystems). PCR cycle parameters were as follows: 95°C for 90 seconds, forty cycles at 94°C for 15 seconds, and 68°C for 1 minute.

### 2.7. Phylogenetic analysis

The *Anelloviridae* family constitutes a highly heterogeneous group of viruses that are classified based on the genetic similarity of the open reading frame 1 (ORF1). The current demarcation criterion for *Anelloviridae* species is set at 35% nucleotide sequence identity of the ORF1 by ICTV taxonomy. We extracted all ORF1 sequences with ORF Finder from the dataset and clustered these at 35% cut-off to generate a non-redundant anellovirus database [21]. Amino acid alignment was performed with MUSCLE in MEGA7.0 [22]. A maximum-likelihood (ML) phylogenetic tree was generated from the extracted ORF1 amino acid sequences in MEGA7.0 with LG + F model. Support for ML trees was assessed by 1000 bootstrap replications.

### 2.8. Statistics

All statistical analyses were performed using R-software [23]. The non-parametric Wilcoxon rank-sum test was used to test statistically significant differences of *Anelloviridae* abundance and sum of species in patients with or without nephrotoxic side-effects. A similar approach was applied for determining 'statistical significance between virome data and post-LT complications. Spearman's rank correlation coefficient was calculated to determine the correlation between the qPCR and metagenomic sequencing results. Fisher's exact test was used to determine significant differences in viral presence and absence count data and categorical variables. The Friedman test was used for repeated measures with a post-hoc Dunn's-test analysis with Bonferroni correction for multiple testing. Kaplan-Meier curves were constructed using the survival package and FactoMineR was utilized to perform factor analysis of mixed data [24,25]. The lme4 package was used for logistic regression analysis [26]. Statistical significance was assessed at an alpha level of < 0.05. The ggplot2 package was used for designing figures [27].

### 2.9. Role of the funding source

The funders had no role in the study design, data collection, data analysis, interpretation, and writing of the report.

## 3. Results

### 3.1. Participants

Twenty-four patients that received a liver transplantation due to HBV related end-stage liver diseases were included in this study and we collected a total number of 142 longitudinal samples. These individuals were part of the chronic HBV cohort that are followed in UZ Leuven, Belgium. Patients were chronically infected with HBV and three were co-infected with hepatitis D virus (HDV) before LT. Both recipient and donor characteristics are summarized in Table 1. The diagnosis for LT in these individuals were either liver cirrhosis ( $N = 10$ ) or hepatocellular carcinoma ( $N = 14$ ) due to a chronic HBV infection. The study population consisted of 19 males and 5 females with a median age of 58 (range, 39–70) at the time of liver transplantation (Table 1). The median lab MELD score was 15 and the median donor age 52. Furthermore, we retrieved blood transfusion data from 23/24 patients. In these 23 patients, a variety of blood product were given during the course of surgery, including erythrocyte concentrates, fresh-frozen plasma and platelet concentrates. No differences were observed in demographic data and patients diagnosed with liver cirrhosis or HCC.

The clinical data revealed that all patients received nucleoside analogues before LT, including lamivudine, tenofovir, entecavir, or adefovir and lamivudine dual treatment. Anti-HBV treatment was complemented with the administration of HBIG for all patients post-LT, which was part of the standard of care protocol for HBV related

**Table 1**  
Demographic overview of the study population. The numbers are expressed as absolute values (percentage) or median (range).

Recipients characteristics				
Gender	Male	19	(79%)	
	Female	5	(21%)	
Age		58	(39–70)	
Disease	Cirrhosis	10	(42%)	
	HCC	14	(58%)	
LabMELD		15	(7–34)	
Antiviral therapy	HBV-nucleoside analogues	24	(100%)	
	Lamivudine	13	(54%)	
	Tenofovir	7	(29%)	
	Adefovir + Lamivudine	3	(13%)	
	Entecavir	1	(4%)	
	HBV Immunoglobulins (post-LT)	24	(100%)	
	CMV-nucleoside analogues	5	(21%)	
	Valganciclovir	5	(100%)	
	HDV coinfection		3	(13%)
	Blood transfusion		23	(96%)
Pre-LT	AST	62	(13–157)	
	ALT	52	(14–115)	
Hospital stay (days)	ICU	5	(1–23)	
	Off ICU	16	(7–43)	
Ischemic perfusion time (hours)	Cold	7	(4–11)	
	Warm	0.7	(0.5–1.3)	
Donor characteristics				
Gender	Male	18	(75%)	
	Female	6	(25%)	
Age		52	(16–57)	

LT. Furthermore, five patients received valganciclovir as anti-CMV treatment. Patients followed a strict therapeutic protocol including immunosuppressive drugs and anti-inflammatory treatment post-LT. This protocol included a standard initiation therapy with tacrolimus, mycophenolate mofetil and methylprednisolone. In three patients that suffered from liver cirrhosis, tacrolimus was combined with everolimus.

### 3.2. Sequencing data

Illumina sequencing generated ~1.4 billion reads, which was reduced by removing low quality and human genome reads to ~0.5 billion reads. In total, 65 million reads were annotated as viral and corresponded to 4.7% of all the generated reads. The majority of viral reads were attributed to eukaryotic viruses (70.9%), followed by prokaryotic viruses (28.9%) and unclassified viral reads (0.2%) (Fig. 1). Three known bloodborne viral families that infect humans were detected, including *Anelloviridae*, *Hepadnaviridae*, and *Flaviviridae*, together with HDV (prototype virus of the deltavirus genus). Different genera of the *Anelloviridae* consisting of alphatorque, betatorque, and gammatorquevirus, as well as a group of unclassified anelloviruses were the most frequently identified viruses and widely present in the blood stream of the study population.

The virome composition was determined in plasma samples at different time points before and after LT. The relative abundance of eukaryotic viral families before (A and B) and after (C, D, E, and F) LT (Fig. 2(a)) were strikingly different, indicating an impact by the initiation of immunosuppressive therapy post-LT. Before transplant (samples A and B), the eukaryotic viral component is majorly composed of the *Hepadnaviridae* family (52%). Sample A corresponds to the first sample that was collected in UZ Leuven after which all patients received anti-HBV therapy. The impact of therapy is illustrated by the decline of *Hepadnaviridae* abundance (24%) seen in sample B, shortly before LT. In total, six and eight patients out of 24 were positive for HBV reads and were detected before transplant in samples A and B respectively. Also, members of the deltavirus genus (HDV in two and

three patients in samples A and B respectively) were detected in three patients. Furthermore, infections with *Flaviviridae* were observed in two patients at timepoint A and one patient in timepoint B, making up 18% and 5% of the relative abundance of all eukaryotic viruses respectively (Fig. 2(b) and (a)).

Towards transplantation, the relative abundance of the *Anelloviridae* increases. While in sample A, the *Anelloviridae* family constituted only 19% of the eukaryotic viral reads, in sample B the abundance increased up to 53%. After transplant (samples C, D, E, and F) the virome is dominated by the *Anelloviridae* (Fig. 2(a) and (b)).

Besides anelloviruses, also *Flaviviridae* were detected with 4% of the annotated reads in both samples D and E, and 7% in sample F. The *Anelloviridae* make up the remaining eukaryotic viral reads. After transplant, the number of patients that were found positive for *Anelloviridae* increased up to 23 out of 24 patients in samples E and F (Fig. 2(b)). Differentiating the study population based on the clinical indication for LT, shows a sustained HBV abundance in HCC patients before transplant while in cirrhotic patients the abundance decreases. However, this trend did not reach statistical significance (Wilcoxon rank-sum:  $p > 0.05$ ).

To further explore the viral presence in liver cirrhosis and HCC patients, we generated cross-tables (Table 2) for the different viral families and patient diagnosis. For the *Anelloviridae*, the patients were categorized based on the tertiles of the average anellovirus abundance in high, intermediate and low burden groups. Although, a Fisher's exact test did not support differences of *Flaviviridae* presence between cirrhosis and HCC patients (Fisher's exact test:  $p > 0.05$ ), an increasing trend was observed in cirrhosis patients. Finally, patients diagnosed with HCC had a higher prevalence of the *Hepadnaviridae* (Fisher's exact test:  $p < 0.05$ ).

### 3.3. *Anelloviridae*

Due to the large abundance of *Anelloviridae* in the transplant patients, we further characterized their genera. The anelloviruses identified in this study were annotated in three genera and an additional group of unclassified anelloviruses. No significant changes were detected in the relative abundance of the three genera (Fig. 3(a)). However, the absolute number of reads of alphatorque, gammatorque and unclassified anelloviruses increased after transplant (Fig. 3(b)). To confirm the NGS results for the anelloviruses, an in-house designed qPCR assay was used on the original samples. A linear correlation was found between the number of reads and the qPCR results (Fig. 3(c), Spearman's rank correlation  $r = 0.85$ ,  $p < 0.001$ ).

Anellovirus ORF1 was extracted from the non-redundant contig dataset to perform phylogenetic analysis. A ML phylogenetic tree was inferred from the aligned amino acid data and shows the clustering of the ORF1 sequences in the three main genera and unclassified *Anelloviridae* sequences (Fig. 4). To determine the presence or absence of the different ORFs in the samples, a threshold of 100 reads was set. An increasing number of anellovirus species were detected in the samples after transplant, with the majority of species derived from the alphatorque genus.

### 3.4. The virome and post-transplant complications

The clinical data revealed incidences of post-LT side-effects in the study population. Primarily, cases of mortality, post-LT infection, nephrotoxicity and new-onset type II diabetes (NO-TIID) were reported (Table 3). The frequency of post-LT complications was compared between cirrhosis and HCC patients. The highest discrepancy was observed in mortality, with six patients in the HCC group compared to one in the cohort of patients diagnosed with liver cirrhosis (Fig. S1). However, no statistically significant differences were observed across the different post-LT complications (Fisher's exact test:  $p > 0.05$ ).

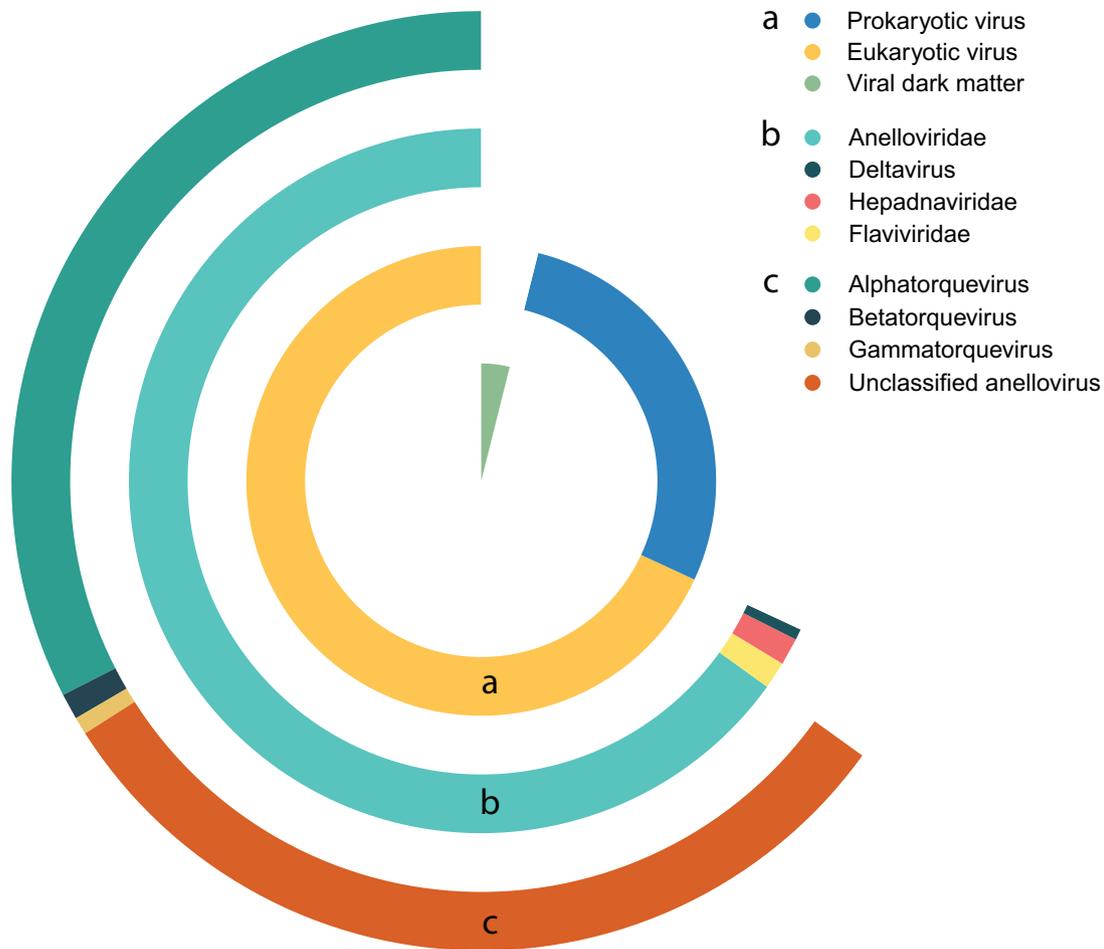


Fig. 1. Distribution of the annotated viral reads over different taxonomical ranks.

### 3.5. Nephrotoxicity

Nine patients (38%) were diagnosed with symptoms of nephrotoxic side-effects post-LT. We compared the number of *Anelloviridae* reads in patients that developed kidney failure to patients that did not report impaired kidney function. The tacrolimus level remained between the reference values (Fig. 5(a)) (5–15  $\mu\text{g/L}$ ) and did not differ between both patient groups. However, both the absolute number of *Anelloviridae* species and the viral abundance indicate a significant increase of this family in patients suffering from nephrotoxic side-effects post-LT (Fig. 5(b) and (c), Dunn's-test). Interestingly, the increase in anellovirus preceded and paralleled increases in serum creatinine concentrations, supporting the association between anellovirus and renal insufficiency (Fig. 5(d)). Besides an increase of the *Anelloviridae* read abundance on family level, both reads attributed to the alphatorque genus and unclassified anelloviruses were higher for patients with renal complications in post-LT samples (E/F and D/E respectively, Dunn's-test). To evaluate the individual *Anelloviridae* species in patients with different clinical courses, we compared the OTU presence in both patient groups (Fig. 5(g) and (h)). This analysis indicated that 8/9 (89%) of patients with nephrotoxic symptoms carried the SEN-virus in timepoint D (Fig. 5(e)), compared to 3/15 (20%) non-nephrotoxic patients (Fig. 5(f)) (Fisher's exact test:  $p < 0.05$ ).

### 3.6. Infections

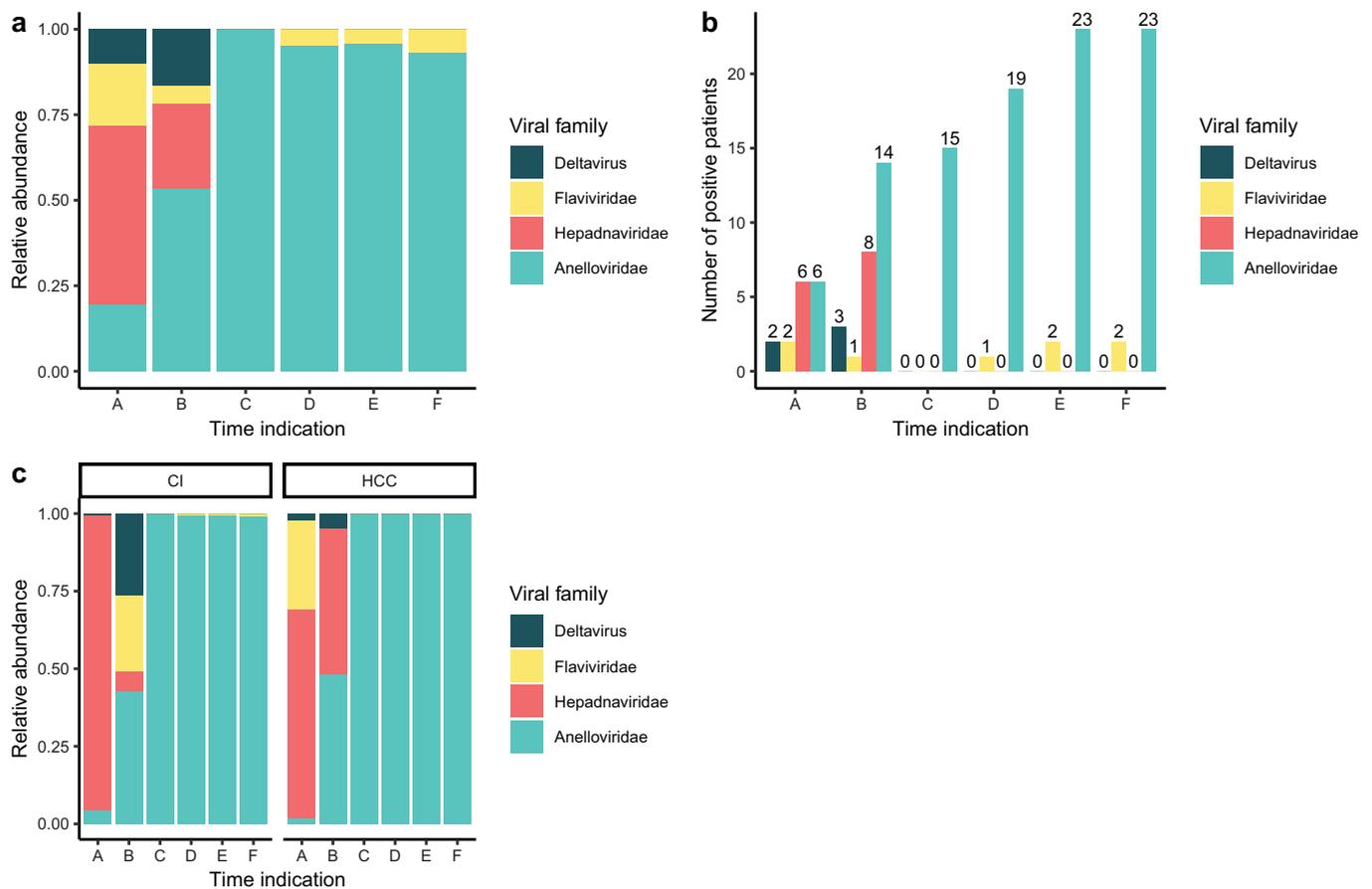
Analysis of routine laboratory results and patients' clinical data revealed a substantial number of infections occurring after LT. A total of 16 (67%) patients experienced flares of infection in post-transplant

samples, either bacterial or viral infections (Fig. 6 and Table 3). Infections were documented during regular and emergency consultations in post-transplant care. We analyzed the anellovirus load in samples close to the diagnosis of infection. The data indicates that in both the number of reads as well as the qPCR data, the *Anelloviridae* abundance is increased during infection compared to non-infection time-point (Wilcoxon rank-sum:  $p < 0.05$ ).

### 3.7. Survival analysis

A 5-year survival analysis was performed to determine the impact of initial diagnosis on post-LT survival (Fig. S2). No significant difference was found in post-LT survival after five years (log rank test:  $p > 0.05$ ). In total, three patients died in the cirrhosis group, with pneumonia being the cause of death in two patients and cancer in one patient. In the group of individuals that received an LT because of HBV induced HCC, six patients died of recurrent HCC or HCC metastasis and one individual of intestinal ischemia. Remarkably, post-LT survival for individuals that died during follow-up was lower for patients with HCC (median survival 24.5 months) compared to cirrhosis patients (median survival 98 months).

A more detailed analysis was performed to study the relationship between viral families and 5-year post-LT survival. All patients had post-LT samples that were positive for anelloviruses through the course of follow-up. Therefore, to study the impact of anellovirus burden on patient survival, we classified the population according to the average post-LT presence of anelloviruses (Fig. S2(b)). Between the different groups (high, medium and low *Anelloviridae* abundance) no difference was found in 5-year post-LT survival (log rank test:  $p >$



**Fig. 2.** (a) Relative abundance of the detected viral families in different timepoint before (A and B) and after (C, D, E and F) liver transplant. (b) Number of patients that are positive for the different viral families before (A and B) and after (C, D, E and F) liver transplant. (c) Relative abundance of the detected viral families in CI and HCC patients. CI: cirrhosis, HCC: hepatocellular carcinoma.

**Table 2**

Cross tables for the presence of viral families in patients diagnosed with liver cirrhosis or hepatocellular carcinoma. Numbers are expressed in frequencies and the percentage of patients positive for the virus within the disease group.

N (%)	Anelloviridae			Hepadnaviridae*		Flaviviridae		Deltavirus	
	High	Medium	Low	Present	Absent	Present	Absent	Present	Absent
CI	4 (40)	3 (30)	3 (30)	1 (10)	9 (90)	3 (30)	7 (70)	1 (10)	9 (90)
HCC	4 (28)	5 (36)	5 (36)	8 (57)	6 (43)	1 (7)	13 (93)	2 (14)	12 (86)

\* Fisher's exact test  $p < 0.05$ .

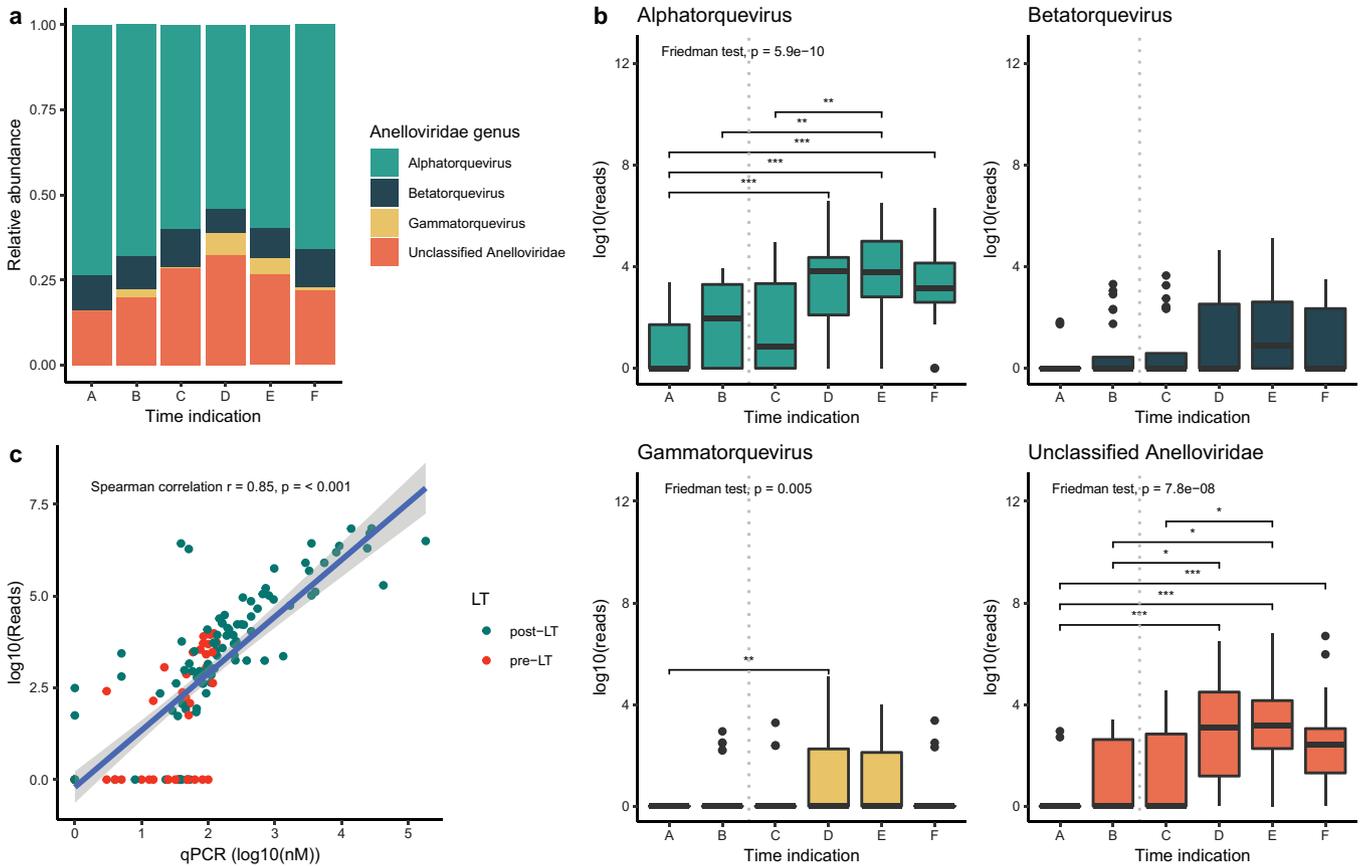
0.05). In patients that were tested positive for HBV in pre-LT samples, 56% of the patients were still alive after five years compared to 80% in HBV negative patients (Fig. S2(c)). Furthermore, HDV co-infected patients ( $N=3$ ) were still alive after five years. Patients that were found positive for HPgV in follow-up samples did not report mortality cases ( $N=4$ ) post-LT (Fig. S2(d)).

### 3.8. New-onset type II diabetes

Besides nephrotoxicity, seven patients (29%) were diagnosed with new-onset type II diabetes (NO-TIID). Tacrolimus concentration in patients were not different in NO-TIID patients (Fig. S3(a), Wilcoxon rank-sum:  $p > 0.05$ ). However, there was an increasing trend in the *Anelloviridae* abundance shortly after LT (Fig. S3(b)). Furthermore, comparing the most abundant OTUs of patients with and without NO-TIID (Figs. S3(c) and (d), respectively) did not show significant changes (Wilcoxon rank-sum:  $p > 0.05$ ).

### 3.9. Clinical relevance of the *Anelloviridae*

To study the relationships between individuals and different quantitative and qualitative variables, a factor analysis of mixed data was applied (Fig. 7). In multiple panels, a clustering of patients diagnosed with post-transplant complications were observed. The two dimensions in the analysis account for 28.8 % of the variance of the dataset. Scattering across the second dimension is primarily driven by clinical markers of hepatitis and the logarithm of the *Anelloviridae* reads in the opposite direction (Fig. 7(a)). Serum AST and ALT ( $R^2$ : 0.79 and 0.71 respectively,  $p < 0.05$ ) are positively correlated and the *Anelloviridae* abundance is negatively correlated ( $R^2$ :  $-0.52$ ,  $p < 0.05$ ) with dimension one. The vertical distribution is mainly related to metastasis and mortality (Fig. 7(d)). Clustering of samples diagnosed with infection and nephrotoxicity along the horizontal axis partially overlap the distribution of the *Anelloviridae* abundance (Fig. 7(a)–(c)). These findings indicate that the *Anelloviridae* abundance might have discriminative power to identify nephrotoxicity and infection in clinical samples.



**Fig. 3.** (a) Relative abundance of the *Anelloviridae* genera before (A and B) and after (C, D, E and F) liver transplantation. (b) Logarithmic value of the absolute number of reads for the different *Anelloviridae* genera before and after liver transplantation. Grey dotted line: liver transplantation. (c) Linear model describing the correlation between NGS and qPCR data for *Anelloviridae* abundance. Red: pre-liver transplantation, blue: post-liver transplantation. Vertical grey dotted line: liver transplantation. Dunn's test: \*  $p < 0.05$  \*\*  $p < 0.01$  \*\*\*  $p < 0.001$ .

To further elucidate the diagnostic potential of the *Anelloviridae* abundance, a univariate logistic regression analysis was applied. The clinical outcome was used as the outcome variable and the logarithm of the *Anelloviridae* reads as the independent variable with patient ID as random effect. We observed a significant odds ratio (OR) for infection (OR 1.58, 95% CI 1.24–2.15, logistic regression:  $p < 0.001$ ) and renal failure (OR 1.59, 95% CI 1.21–2.20, logistic regression:  $p < 0.01$ ) (Table 4). However, no significant OR was observed for mortality (OR 1.04, 95% CI 0.11–11.70, logistic regression:  $p > 0.05$ ) and NO-TIID (OR 1.63, 95% CI 0.93–3.47, logistic regression:  $p > 0.05$ ).

#### 4. Discussion

Metagenomic studies have provided a strong link between the clinical status of patients and fluctuation of microorganism populations living and circulating in different biological niches of the human body [4,28]. This research added fundamental knowledge for finding disease aetiology, therapeutic and prophylactic measures. For the first time, we studied the fluctuation of the blood virome in LT recipients before and after surgery in a set of longitudinal samples.

##### 4.1. Viral persistence (HBV)

The implemented antiviral strategy to prevent resurgence of HBV infection seemed to be effective considering that no HBV was detected post-LT in the study population. The majority of patients positive for HBV were diagnosed with HCC. Among these individuals, the primary cause of mortality was related to recurrent HCC or HCC metastasis. This supports previous data describing HBV related HCC

as a risk factor for post-LT mortality [29,30]. The increased presence of HBV in HCC patients could be explained by the decreased availability of hepatocytes for viral replication in liver cirrhosis patients [31]. Also, highly active tumour cells in HCC conditions could function as a viral replication site and act as a potential reservoir [32]. However, providing HBV prophylaxis reduces the risk of recurrent HBV infection as demonstrated in this study.

##### 4.2. Post-LT complications

An increase in the abundance and number of *Anelloviridae* species was observed post-LT. Furthermore, multivariate and univariate analyses further confirmed the potential clinical relevance of the *Anelloviridae* abundance in diagnosing post-LT complications. These observations corresponded with the initiation of immunosuppressive therapy. The interaction between immunosuppression and blooming of the anellovirus population in LT recipients approves previous studies in other organ transplantation settings [4,33].

The *Anelloviridae* family constitutes a highly heterogeneous group of viruses that are widely distributed in healthy individuals [34]. Anellovirus infection is acquired shortly after birth from which it establishes a possibly lifelong infection. Currently, no pathogenic effects have been subscribed to these viruses, albeit an association with autoimmune diseases and cancers has been implied in the past [35].

Previous studies demonstrated an association between the anellovirus load and solid organ rejection [4]. Of note, a lower anellovirus abundance corresponded to an increased risk of organ rejections, while a high *Anelloviridae* load translated in an elevated susceptibility

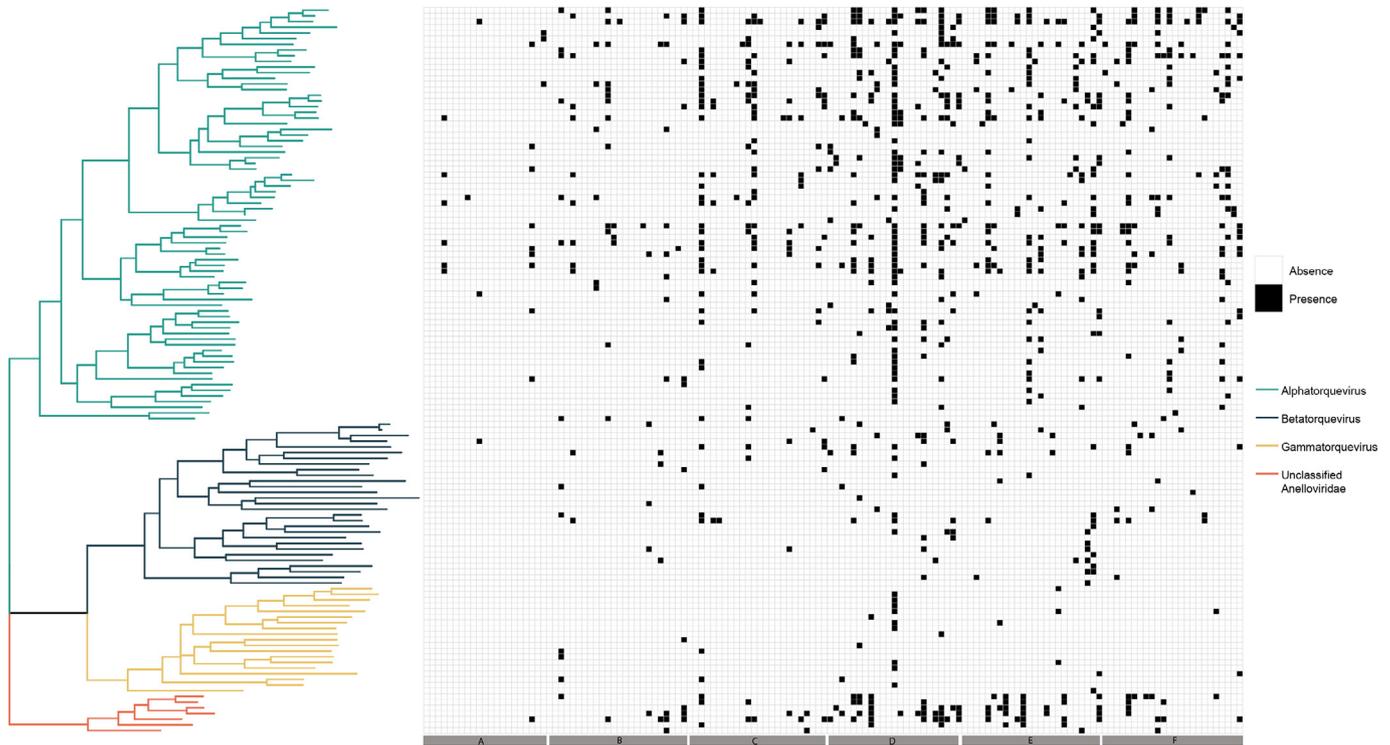


Fig. 4. ML phylogenetic tree of the extracted amino acid ORF1 sequences with a corresponding presence and absence heatmap ranked according to the different timepoints.

**Table 3**  
Overview of the post-liver transplant complications.

Variable	N	Percentage
Mortality	<b>7</b>	<b>(29)</b>
Recurrent HCC	2	(29)
HCC metastasis	4	(57)
Pneumonia	1	(14)
Infection	<b>16</b>	<b>(67)</b>
Viral		
CMV	2	(11)
EBV	2	(11)
Polyomavirus	1	(5)
Bacterial		
Urinary tract	7	(37)
Positive blood culture	4	(21)
Gastroenteritis	2	(11)
Nephrotoxicity	<b>9</b>	<b>(38)</b>
NO-TIID	<b>7</b>	<b>(29)</b>

of opportunistic infections and secondary cancers. Although, no cases of liver transplant rejection were observed, 23/24 patients suffered from post-LT complications.

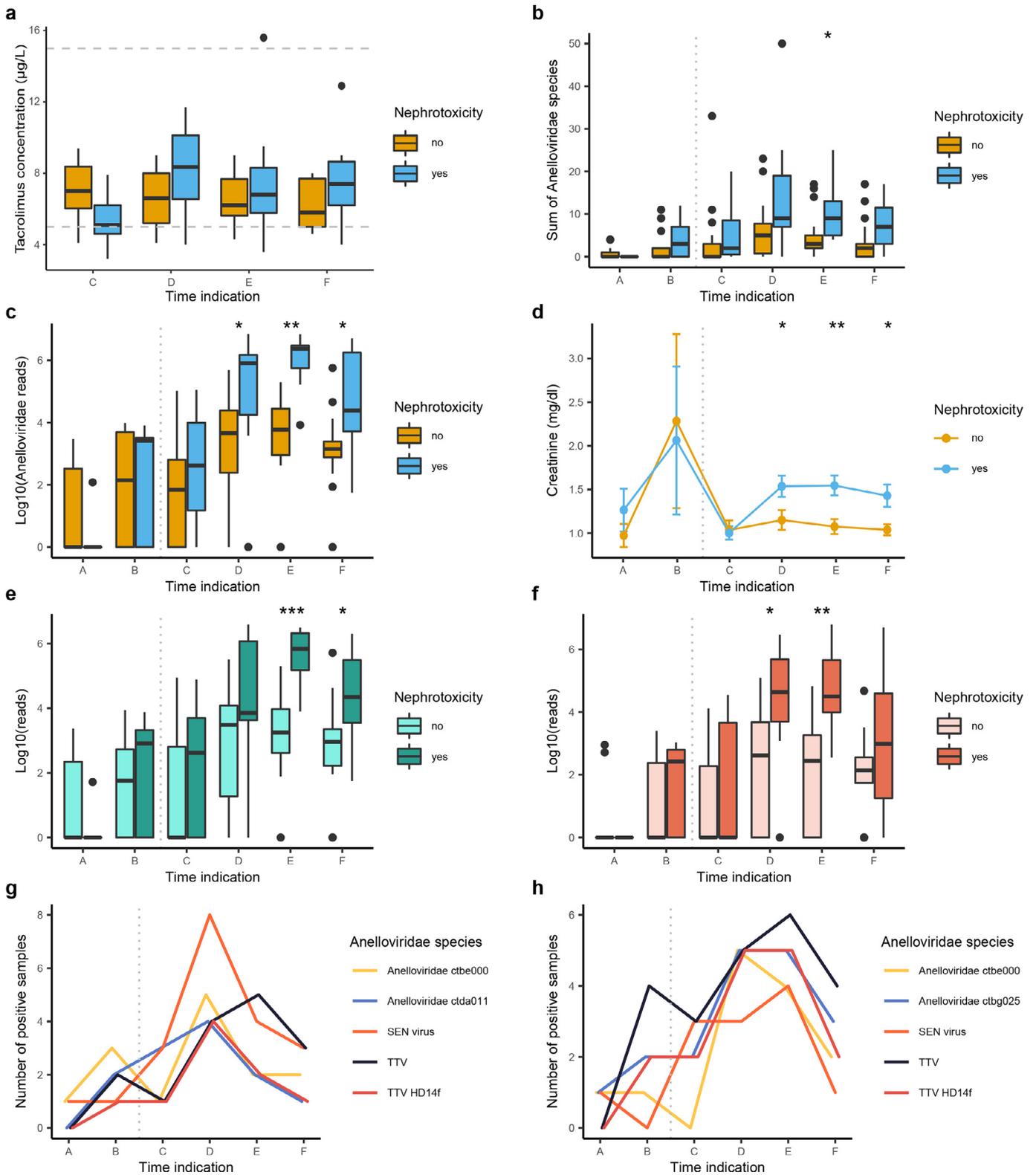
#### 4.3. Post-liver transplantation infections

In 16 patients we observed bacterial or viral post-transplant infections. The risk of infection is the result of two factors, the pathogenic exposure and the patient immune status [5]. Bacterial infections that occurred shortly after transplantation were mainly related to surgical procedures and hospital environment exposure, i.e. nosocomial exposure. The reduced immunocompetence after samples C ( $\pm$  3-6 months post-LT) accommodated the occurrence of opportunistic viral infections, such as CMV and EBV reactivation. Importantly, the samples that were collected during the course of infections, contained a higher *Anelloviridae* load, both in terms of read number and qPCR value (Wilcoxon rank-sum:  $p < 0.05$ ). This data suggests that

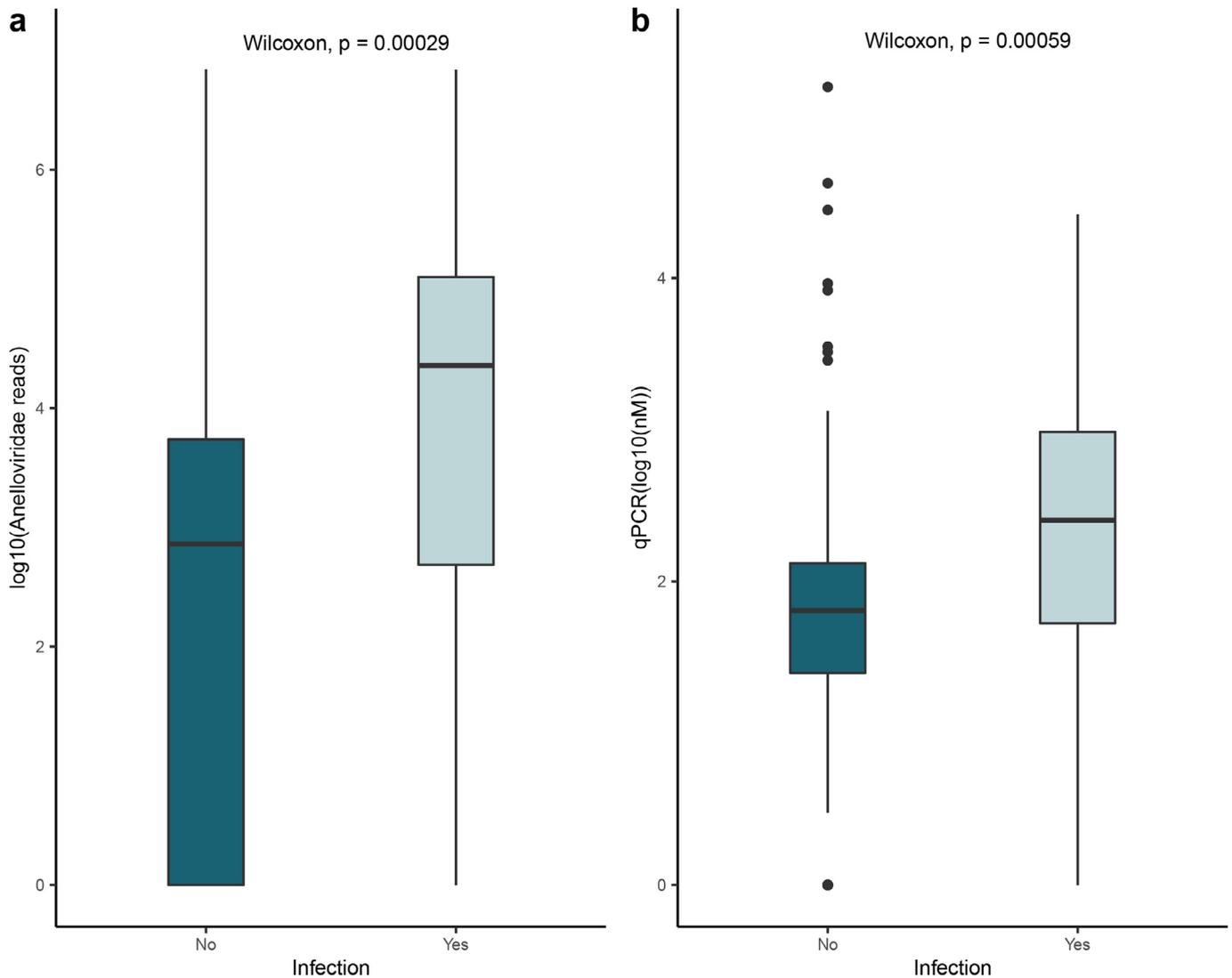
anellovirus kinetics might be indicative for post-transplant infections, which is consistent with previous reports in different types of organ transplantations [4,36,37]. In contrast to previous findings, this study focussed at intact viral particles in an untargeted manner, that allows a broader exploration of the viral landscape in LT patients. Therefore, a more detailed assessment of the wide variety of anelloviruses in immunosuppressed patients was achieved covering multiple genera of the *Anelloviridae* family. In current clinical practice, the reduced signs of inflammation and possible drug interactions complicate the development of optimized diagnostic and treatment strategies in immunocompromised patients respectively [5]. Our findings support the potential use of anelloviruses as a surrogate marker of the net immune status in immunocompromised patients [4,38]. However, these findings should be interpreted with caution, as not all infectious episodes were accompanied by high *Anelloviridae* loads and vice versa. A careful longitudinal follow-up of organ transplant patients is required to validate the diagnostic relevance of anelloviruses in post-transplant care.

#### 4.4. Post-liver transplantation nephrotoxicity

Besides post-LT infections, nine patients suffered from nephrotoxic side-effects after liver transplantation. An impaired renal function was related to a higher anellovirus load after LT compared to non-renal failure patients. Multiple scenarios might explain this clinical observation. The administration of immunosuppressive calcineurin inhibitors (CNIs), like tacrolimus, have significantly improved transplantation success and patient survival. However, nephrotoxicity is a common side-effect of long-term exposure to these compounds [39]. Therefore, the reported increase of *Anelloviridae* in patients with an impaired kidney function can be a direct consequence from the elevated tacrolimus concentration, which also negatively affects renal function. However, no significant differences were observed in the plasma tacrolimus concentrations in patients with or without renal toxicity. In contrast, the *Anelloviridae* load



**Fig. 5.** (a) Distribution of the tacrolimus concentration in patients diagnosed with or without nephrotoxic side-effects. (b) Sum of all *Anelloviridae* species divided in patients with and without nephrotoxic side effects. (c) Differences in the number of *Anelloviridae* reads (logarithmic) between patients with and without nephrotoxic side effects. (d) Differences in blood creatinine concentrations between patients with and without nephrotoxic side effects. (e) The number of reads attributed (logarithmic) to the alphatorque genus. (f) The number of unclassified *Anelloviridae* reads (logarithmic). (g) The most abundant viral species in patients with nephrotoxic side-effects. (h) The most abundant viral species in patients without nephrotoxic side-effects. Blue: with nephrotoxic side effects, yellow: without nephrotoxic side effects. Vertical grey dotted line: liver transplantation. Horizontal grey dotted line: reference values. Wilcoxon rank-sum test: \*  $p < 0.05$  \*\*  $p < 0.01$  \*\*\*  $p < 0.001$ .



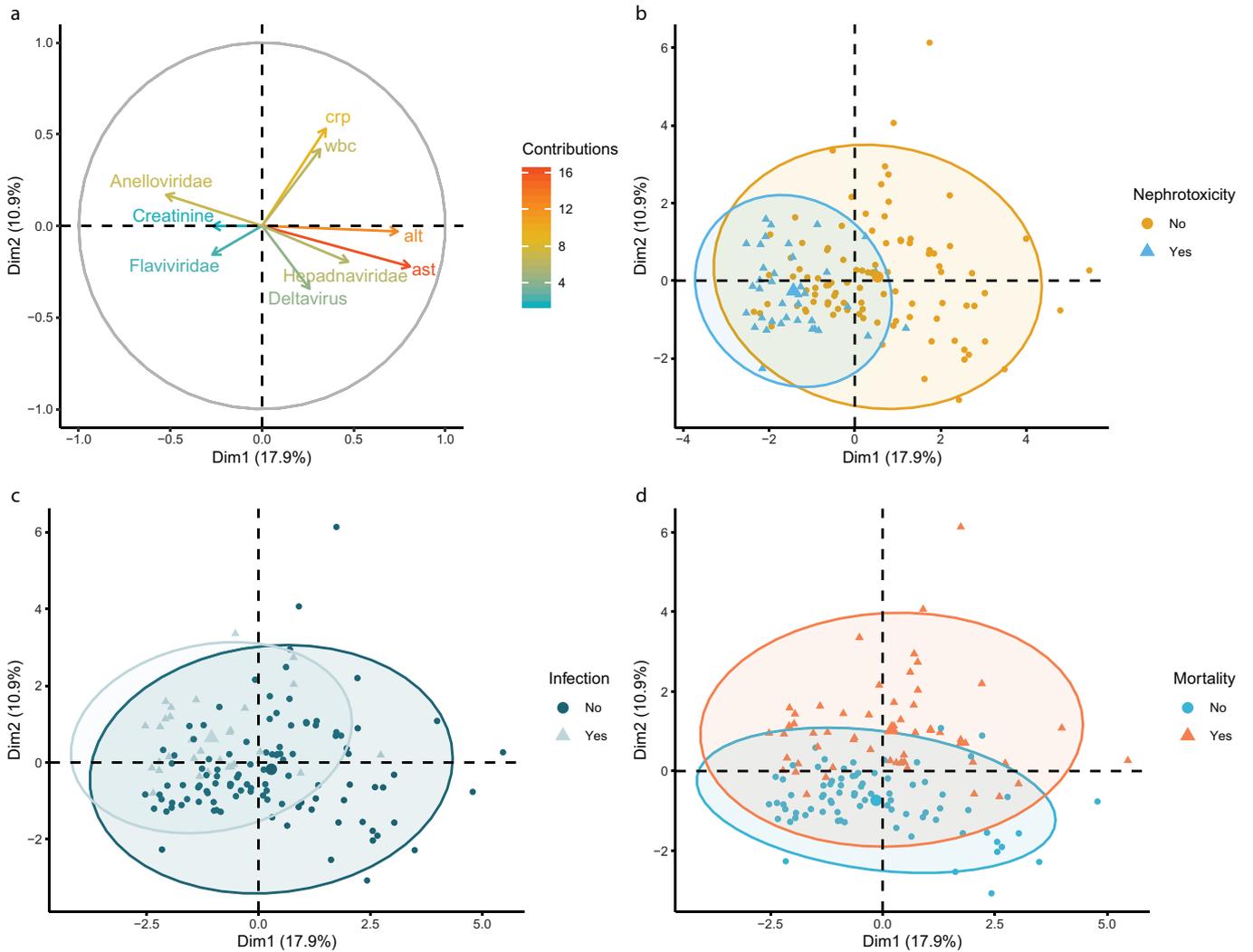
**Fig. 6.** (a) The *Anelloviridae* reads abundance in samples diagnosed with or without infections. (b) qPCR results of the *Anelloviridae* family in samples diagnosed with or without infections. Only significant *p*-values are shown (Wilcoxon rank-sum test).

corresponded to an increase in the plasma creatinine concentration. Creatinine clearance is a biomarker that indicates kidney functioning [40]. Although, it is not considered a highly sensitive biomarker, our data demonstrates that serum creatinine is elevated in patient with renal failure. Furthermore, the data suggests that the *Anelloviridae* could be used as a proxy for nephrotoxic side-effects, based on the increased abundance in patients suffering from renal failure. A more detailed analysis of the individual anellovirus species revealed that some species occurred more often in patients suffering from renal failure than others. As such, SEN-virus was detected in 8/9 timepoint D samples for patients with reduced renal function. SEN-virus infection has been related to non-A-E hepatitis, albeit the data is highly inconsistent [41]. Considering the epidemiological pattern of this virus in different populations, it is unlikely that it exerts detrimental consequences for the host. Studies that demonstrated the presence of SEN-virus in healthy blood donors imply that this virus can be transmitted through blood transfusions [42,43]. However, the viral abundance did not correspond to the frequency or type of blood transfusions. Another explanation for the increase of SEN-virus post-LT, could be related to the increased *Anelloviridae* abundance in immunosuppressed conditions. Therefore, the incremental

replication causes the viral load to pass the detection threshold of NGS post-LT. Future studies should focus at characterizing the dynamics and elucidating the role of SEN-virus in post-transplant complications and blood transfusion practice.

#### 4.5. Post-liver transplantation new onset type II diabetes

Besides the emergence of nephrotoxic side-effects, a substantial number of patients reported post-LT NO-TIID (7/24). Multiple factors are known to contribute to the development of NO-TIID like pre-existing insulin resistance. Furthermore, drugs administered in post-LT standard care regimen negatively affect glucose control of the patient and instigates the progression to NO-TIID [44]. For instance, corticosteroids are widely used in high doses shortly after transplant and are known for causing hyperglycaemia. However, tapering of the drug improves glucose tolerance and restores the normal homeostasis. Besides corticosteroids, CNIs (e.g. tacrolimus) have been associated with the development of NO-TIID. In this study, we analysed *Anelloviridae* kinetics in patients with or without NO-TIID. We detected an increasing trend in the *Anelloviridae* abundance in NO-TIID patients, albeit no statistically significant result was found. Also,



**Fig. 7.** (a) Contribution plot of the quantitative variables across the first two dimensions. (b) Clustering of individual samples of patients diagnosed with nephrotoxicity. (c) Clustering of individual samples of patients diagnosed with post-liver transplantation infections. (d) Clustering of individual samples of patients that died during follow-up.

**Table 4**  
Relationship between *Anelloviridae* abundance and post-liver transplant complications.

		Odds ratio	95% CI
<i>Anelloviridae</i>	Infection	1.58**	1.24–2.15
	Nephrotoxicity	1.59*	1.21–2.20
	Mortality	1.04	0.11–11.70
	NO-TIID	1.63	0.93–3.47

Logistic regression analysis

\*  $p < 0.01$

\*\*  $p < 0.001$

NO-TIID did not seem to correlate with other post-LT outcomes, in contrast to previous findings [45].

The plasma concentration of CNIs is an important indicator of immunosuppression and possible risk of side-effects [46]. However, we demonstrated that the level of CNIs did not discriminate between individuals diagnosed with or without post-LT complications. Although, CNIs are considered as the main contributor to post-transplant toxicities, a possible synergistic scenario between immunosuppressive drugs and viral families have not been evaluated. For instance, it has been shown that viruses such as the BK polyomavirus are involved in renal failure of non-renal solid organ transplant recipients who received immunosuppressive agents [47]. Although, we

did not report the presence of BK polyomavirus in our samples, the clinical impact of other viral families found in this study should be further explored.

#### 4.6. *Anelloviridae*

The majority of clinical studies have focused on the presence the alphatorquevirus genus of the *Anelloviridae* family. The alphatorquevirus genus is often targeted by qPCR in different clinical manifestation and is considered a potential marker for immunocompetence in organ transplant patients [48,49]. Although, this approach seems promising, a well-validated anellovirus biomarker is still lacking. Our findings indicate that a single patient can be coinfecting with multiple species that belong to different genera of the *Anelloviridae* family. This complicates the development of a unique biomarker for clinical interpretation. Future studies should regard the high heterogeneity of the *Anelloviridae* family to validate the clinical importance of these viruses as biomarkers for immunocompetence.

#### 4.7. *Human pegivirus*

Besides anelloviruses, we detected human HPgV in samples of four patients (three patients with cirrhosis and one patient with HCC). All patients that were found positive for HPgV were still alive 5-years post-LT, which might indicate a protective role of HPgV in

post-LT survival. HPgV infection has received a growing attention by the research community because of its immune modulating potential and positive outcomes in other viral infections. Patients coinfecting with HIV and HPgV demonstrated a prolonged survival with decreased HIV viral load and higher CD4+ T-cell counts [50–52]. Furthermore, HIV infected patients that acquired HPgV through blood transfusion demonstrated an improved survival compared to non-HPgV infected patients [53]. These characteristics have inspired researchers to explore the use of HPgV as a bio vaccine to complement anti-HIV therapy [11,54]. Besides the positive impact on HIV-outcome, HPgV co-infection demonstrated a potential protective role in Ebola virus infected patients [55]. Despite a single study that reported a decreasing trend in HBV DNA in HBV and HIV coinfecting individuals with HPgV, the role of HPgV in HBV infected individuals is still unexplored [56]. Furthermore, the impact of HPgV and post-LT mortality should be further investigated in future studies. In contrast to the positive impact on HIV and Ebola patients' survival, the immune modulating effect of the virus may contribute to the development and progression of non-Hodgkin's lymphoma [57,58]. Therefore, additional research is warranted to study the long-term impact of HPgV infection in healthy, HBV infected and organ transplanted patients in a larger population.

In this study, the samples were collected in a retrospective manner from the HBV cohort in UZ Leuven with a limited population size. The relatively small number of patients could have underpowered parts of the analysis. Therefore, future research should confirm the observed clinical relevance of the *Anelloviridae* abundance in a larger population. Furthermore, exploring the virome in different clinical settings is recommended. For instance, studying the virome in other organ transplant or blood transfusion settings will provide a more detailed insight in virome-modulating factors that could attenuate the relationship between these viruses and clinical outcomes.

In conclusion, in the assessment of longitudinal plasma samples, multiple viral families have been detected in the blood virome of liver transplant recipients. The metagenomic approach provided an unprecedented insight in the viral communities. These findings support that the clinical status and pharmacological perturbation of the immune system can have a profound impact on the presence and abundance of viral populations. The notion of an increased *Anelloviridae* abundance and diversity in patients suffering from post-LT side-effects calls for a further exploration of the relationship between these viruses and immunocompromised individuals. The identification of HPgV in a subgroup of our study population, highlights the necessity of studying the impact of this virus on the clinical outcome of HBV infected patients.

#### Data sharing statement

Raw reads were deposited in NCBI's Sequence Read Archive (SRA) database under accession no. PRJNA660895.

#### Declaration Competing Interest

We declare no competing interests.

#### Author contributions

MRP and MVR designed the study. MT, MRP, WL and FN recruited the patients, collected the samples and clinical data. MT and CKY did the NGS sequencing. MT, LB and WD analysed the NGS data. MT did the statistical analysis over the clinical data and NGS findings. MT and FT provided the first draft of manuscript and developed it. LB, WD, CKY, FN, MRP and MVR edited the manuscript and improved it. All authors read and approved the final version of the manuscript.

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#### Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ebiom.2020.103009.

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