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TTV Species Diversity as a Novel Biomarker of Immune Dysregulation in Aging

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ABSTRACT

Torquetenovirus (TTV) is a prevalent virus whose clinical significance remains unclear, potentially linked to immunosenescence. This study examines TTV species in relation to immune impairment, inflammation, and cellular stress response in aging. A subset of recruited age-stratified individuals (RASIG) from the MARK-AGE study was divided into three groups: Cohort 1 A (healthy young adults), Cohort 1B (older adults with mild immune decline), and Cohort 1 C (older adults with marked immune impairment). Analyses included TTV load, species diversity, lymphocyte subpopulations, inflammatory markers, Poly-(ADP-ribose) polymerase (PARP-1) expression/activity. Alpha- and beta-diversity analyses showed the highest

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TTV species diversity in Cohort 1 C, with significant cohort-dependent differences and partially cohort-specific clustering patterns. Increased TTV species number correlated with higher TTV load, elevated CMV IgG levels, and greater immune impairment risk. Specific TTV species were associated with CD4/CD8, and reduced T-cell receptor excision circles, suggesting impaired T-cell homeostasis. TTV viremia positively correlated with C-reactive protein (CRP) and α2-macroglobulin. PARP-1 expression and activity increased in individuals with higher TTV diversity, particularly in the presence of TTV9 and TTV20. TTV load and species diversity are associated with immunosenescence, inflammation, and PARP-1 activation suggesting their potential as biomarkers of age-related immune decline. Longitudinal studies are needed to clarify underlying mechanisms.

1 | Introduction

Anelloviruses, which constitute one of the most prevalent components of the human virome, have the potential to serve as a reliable, accurate, universal, and noninvasive biomarker for evaluating immune system responsiveness. This is particularly significant in geriatric clinical contexts, such as assessing frail elderly patients [1–3]. Currently, there is no universal biomarker to quantify immune system function. The most promising candidate for immunological surveillance is Torquetenovirus (TTV), an anellovirus associated with immunosuppression and mortality in older adults [1, 4, 5]. First discovered in 1997 in a Japanese patient with post-transfusion hepatitis of unknown etiology [6], TTV has since been detected in over 95% of individuals worldwide [7, 8]. Typically, the TTV genome is circular and composed of single-strand DNA, approximately 3800 nucleotides in length. It is organized into a coding region sequence (CDS) of around 2.6 kilobases (kb) and a highly conserved noncoding region (UTR) of about 1.2 kb [9]. The CDS presents at least four partially overlapping open reading frames (ORF1–4) [10].

Despite its high prevalence, the virus has not been directly linked to any specific disease. However, TTV loads can vary widely, ranging from 10 to 10 [8] DNA copies per mL of plasma, and tend to increase with age [1, 11]. Since its replication is tightly regulated by the host immune system, TTV may serve as a potential biomarker for immune competence and dysregulation [12–14]. High TTV levels have been observed in various clinical settings, including solid organ and hematopoietic stem cell transplantation, HIV infection, severe sepsis and COVID-19 infection [13, 15–19]. In addition, elevated plasma TTV DNA levels have been reported in Crohn′s disease patients, where they correlated with reduced CD3+ and CD4 + T cells, and in solid organ transplant recipients, where TTV load increased following CMV reactivation. These findings further support the value of TTV viremia as a biomarker of immune dysfunction across diverse clinical contexts [20, 21]. In older adults, higher TTV viremia has been associated with increased mortality risk, physical and cognitive frailty, and ischemic heart disease, suggesting a potential role in immunosenescence [1–3, 8]. Over the years, advances in sequencing technologies have led to the identification of multiple TTV species and genotypes, classified according to genetic diversity. The clinical significance of this diversity remains largely unexplored; however, it is possible that distinct TTV species interact differently with the host immune system and may be differentially associated with immune dysfunction and aging [1, 8, 9, 11]. Aging is accompanied by profound changes in immune function, collectively referred to as immunosenescence. This process is characterized by thymic involution, reduced naïve T-cell production, accumulation of memory T cells, and a chronic low-grade inflammatory state known as “inflammaging” [22]. Thymic function can be assessed by measuring T-cell receptor (TCR) excision circles (TREC), small circular DNA episomes generated during TCR gene rearrangement in thymic precursors. TREC levels decline with age and can be further reduced in certain viral infections [23, 24]. These alterations contribute to an increased susceptibility to infections, reduced vaccine responsiveness, and a higher incidence of age-related diseases. Given the dynamic interaction between TTV and the immune system, studying the distribution of TTV species in aging populations could provide novel insights into the role of the virome in immunosenescence and its potential as a biomarker for immune health.

According to the most recent classification approved by the International Committee on Taxonomy of Viruses (ICTV) (2024 Release, EC 56, Bari, Italy, August 2024), the *Anelloviridae* family is placed within the realm *Monodnaviria*, kingdom *Shotokuvirae*, phylum *Commensaviricota*, class *Cardeaviricetes*, and order *Sanitavirales*. This large and diverse family currently comprises 37 genera and 243 recognized species. Within this family, the genus *Alphatorquevirus* includes 20 human-infecting species (formally designated *Alphatorquevirus homini*), defined on the basis of a 69% sequence identity threshold in the ORF1 coding region [25, 26]. These species are commonly referred to as TTV, namely TTV1, TTV2, TTV3, TTV4, TTV5, TTV6, TTV7, TTV9, TTV10, TTV13, TTV14, TTV15, TTV17, TTV18, TTV19, TTV20, TTV21, TTV26, TTV29, and TTV31. This genetic diversity influences the virus′s interaction with the host and may contribute to its potential association with various diseases [27, 28]. Interestingly, recent studies have highlighted the role of PARPs in host defenses against viruses, either by direct antiviral activity, targeting certain steps of virus replication cycle, or indirect antiviral activity, via modulation of the innate immune response [29]. Given that PARP-1 is a key regulator of cellular stress responses and inflammatory signaling during viral infections, its expression and activity may reflect broader host–virus interactions in aging individuals [30]. Thus, exploring PARP-1 activation in relation to TTV species diversity may provide additional insight into the molecular mechanisms underlying immunosenescence.

In this study, using samples from the MARK-AGE population, TTV viremia, species composition, immune markers, and inflammatory parameters were analysed across individuals with varying degrees of immune competence. The objective was to assess whether TTV species diversity correlates with immune

aging and to explore its potential as a biomarker for immunosenescence and inflammation-driven aging processes. The findings indicated that TTV species diversity increases with age and immune impairment, showing a direct association between the number of viral species, viral load, and immunological alterations. This expands the evidence that TTV can serve as a marker of immune status, particularly in aging and immunosenescence.

2 | Methods

2.1 | Studied Population

As part of the VIROMA study (CE INRCA 23011, 12.04.2023), we quantified TTV viremia and identified TTV species in peripheral blood samples from 300 randomly recruited age-stratified individuals (RASIG) enrolled in the MARK-AGE project [31, 32], a large-scale European project aimed at identifying biomarkers of human aging, including immune-related markers. Subjects who were seropositive for human immunodeficiency virus (HIV), hepatitis B virus (HBV) (except those vaccinated), or hepatitis C virus (HCV) were excluded. Details on recruitment procedures, data collection (anthropometric, clinical, demographic), and laboratory assays have previously been described [33, 34]. The selected cohort had a mean age of 61.2 ± 13.3 years.

Participants were categorized into three groups with similar sex distribution based on immune function. These groups were ranked from 1 to 3 (from highest to lowest immune response) according to previous data and literature:

- Cohort 1 A: healthy younger adults (35–45 years) with no current medication and an intact immune response (CD4/CD8 > 1, low CMV IgG levels, defined as values below the cohort median, and no signs of increased inflammation);
- Cohort 1B: older adults (65–75 years) with mildly reduced but functional immune response, without evidence of an immune risk phenotype (CD4/CD8 > 1, low CMV IgG levels, low inflammatory markers, defined as values below the cohort median (*e.g.*, C-reactive protein (CRP) and neutrophil-to-lymphocyte ratio (NLR)).
- Cohort 1 C: older adults (65–75 years) with an impaired immune response and/or signs of immunosenescence (CD4/CD8 < 1, high CMV IgG levels, elevated inflammatory markers (*e.g.*, C-reactive protein (CRP) and neutrophil-to-lymphocyte ratio (NLR)).

From the MARK-AGE samples stored at IRCCS INRCA, 100 sex-balanced blood samples were chosen from the three groups. After TTV viremia quantification, individuals with a viral load greater than 3.0 Log copies/mL were selected for TTV species characterization using next-generation sequencing (NGS). The study cohort included 38 participants in Cohort 1 A, 46 participants in Cohort 1B, and 58 participants in Cohort 1 C.

Fresh whole blood was collected after overnight fasting. Plasma, serum, peripheral blood mononuclear cells (PBMCs), and whole blood samples were transported from recruitment centers to the MARK-AGE Biobank at the University of Hohenheim, Stuttgart, Germany. Coded samples were then shipped on dry ice to IRCCS INRCA in Ancona, Italy, where they were stored at −80°C until analysis [33].

2.2 | TTV DNA Detection and Quantification

Viral DNA was extracted from whole blood samples using QIAamp DNA Blood mini kit (Qiagen GmbH, Germany) according to the manufacturer′s instructions. TTV DNA presence and load were assessed using a single-step, in-house TaqMan PCR assay, as described elsewhere [8]. The assay, referred to as "universal PCR," employs forward and reverse primers targeting a highly conserved segment of the viral genome′s UTR and quantifies the total TTV DNA load without distinguishing between TTV species present in a single subject′s blood. The lower detection limit was 1.0 Log copies of TTV DNA per mL of blood. Methods for copy number quantification, as well as evaluations of specificity, sensitivity, intra- and inter-assay precision, and reproducibility, have been previously outlined [35].

2.3 | TTV Species Characterization Using TTV-NGS

A whole-genome, amplicon-based, NGS technique was used to detect individual TTV species, as previously described [36, 37]. The selection criteria were a TTV load higher than 3.0 Log copies/mL. Briefly, rolling circle amplification (RCA) technology was used to enrich the circular DNA of the anelloviruses as previously described [36, 37]. The resulting product was quantified using Qubit™ according to dsDNA Quantification Assay Kits (Thermo Fisher Scientific) and diluted for use as a template in the universal anellovirus inverse (UAvI) PCR.

The UAvI PCR was then performed to obtain a genome amplicon of all TTV species [36]. The PCR product was purified and quantified. Libraries were generated using the Illumina DNA Prep kit, with an initial DNA input of 150 ng. The pool of libraries was quantified using Qubit™, and the sizes of the fragments were estimated using the 4200 TapeStation system (Agilent). The denatured libraries were then sequenced using an MiSeq V3 cartridge (600 cycles) (Illumina, San Diego, CA, USA) and NextSeq. 500/550 Mid Output Kit v2.5 (300 Cycles) (Illumina, San Diego, CA, USA), both with a 2×150 bp layout.

2.4 | Bioinformatical Analysis

Sequence analysis was carried out with the bioinformatical pipeline described and performed by Spezia et al. (2023) [37]. In brief, analysis workflow integrated both *de novo* assembly and reference-guided mapping approach. Initially, Fastp (v0.220) was employed to trim low-quality reads and remove adapter sequences, after which Kraken 2 (v2.1.2) filtered out reads of human origin using the GRCh38.p13 reference genome. Following the removal of human sequences, the remaining reads were processed along two complementary paths: they were

assembled *de novo*, retaining only genomes within the expected size range (2800–4000 nucleotides) and subsequently classified via BLASTn according to ICTV 2021 taxonomy parameter criteria, while in parallel they were mapped against an in-house curated anellovirus database (updated to January 2024). Duplicate sequences exhibiting over 90% identity were then clustered using CD-HIT (v4.8.1) to eliminate redundancy. Finally, to cross-validate the assembly, nonhuman reads were re-mapped with BWA-MEM (v0.7.17), and the ORF1 gene sequences identified via orfipy (v0.0.4) were manually reviewed for quality control and accurate classification.

2.5 | PARP-1 Gene Expression

Total RNA was isolated from peripheral blood mononuclear cells (PBMCs) using the RNeasy Mini Kit (Qiagen), and reverse transcription was performed with 0.5 μg of RNA using the SuperScript VILO cDNA Synthesis Kit (Invitrogen, MA, USA). PARP-1 mRNA expression was quantified by real-time PCR using the TaqMan Gene Expression Assay (Applied Biosystems; Hs00242302_m1) on the iCycler iQ detection system (Bio-Rad, Hercules, CA, USA), according to the manufacturer's protocol. Amplification efficiency was assessed using twofold serial dilutions of randomly selected cDNAs (50 to 3.125 ng), with efficiencies between 90% and 100% and correlation coefficients above 0.99. Each sample was analyzed in triplicate using 30 ng of cDNA. Gene expression was calculated using the comparative Ct method, normalized to β-glucuronidase (GUSB; Hs99999908_m1). An inter-run calibrator derived from HCT116 cell cDNA was included in each PCR plate.

2.6 | Determination of PARP Activity in PBMCs

PARP activity was measured immediately after thawing PBMCs using a flow cytometry fluorescence-activated cell sorting (FACS)-based technique [38] with slight modifications. PBMCs were fixed in ethanol, permeabilized, and incubated in reaction buffer. Cellular poly(ADP-ribosyl)ation capacity was induced by adding NAD^+ and an activator oligonucleotide. Cells (2×10^5) were incubated on ice with reaction buffer and activator oligonucleotide (total 100 μL), then incubated at 37°C for 4 min. After centrifugation, cells were resuspended in PBS, and the reaction was stopped with ice-cold ethanol (−20°C, 15 min). Following further centrifugation and washing in FACS buffer, cells were incubated overnight at 4°C with a primary antibody (1:300), then with an Alexa 488-conjugated secondary antibody (1:1000) at room temperature for 30 min. After final washing and resuspension in FACS buffer, cells were kept on ice for flow cytometry analysis. Cell samples were analyzed by flow cytometry in a FACS Calibur II (Becton Dickinson Immunocytometry Systems). A total of 10,000 event files for each sample were acquired individually in "live-gate" mode. Data are expressed as mean fluorescence intensity (MFI) above background.

2.7 | CMV IgG Antibody Titer

CMV-specific IgG in serum were measured using DRG Cytomegalie Virus (CMV) IgG ELISA Kit according to the manufacturer's specifications (DRG International Inc., U.S.A.). CMV IgG levels > 11 U/mL were considered positive.

2.8 | Cell Phenotyping

Lymphocyte subsets in PBMC samples from the MARK-AGE Biobank were analyzed by FACS. Briefly, the BD Multitest IMK kit (340503) was used to identify and quantify mature human lymphocyte subsets, including T lymphocytes (CD3 +), B lymphocytes (CD19 +), helper T cells (CD3 + CD4 +), cytotoxic T cells (CD3 + CD8 +), and natural killer (NK) cells (CD3 − CD16+ and/or CD56 +). Sample acquisition and analysis were performed on a FACSCalibur (Becton Dickinson, Warsaw, Poland) using CellQuest and Multiset software (Becton Dickinson).

2.9 | Statistical Analysis

Subject characteristics are presented as mean ± standard error of the mean (SEM) for continuous variables and as percentages for categorical variables. For continuous variables, normality was assessed using the 1-sample Kolmogorov–Smirnov test. Variables that did not follow a normal distribution were log-transformed, and their normality was re-evaluated after the transformation. Differences among groups were analyzed using One-way Analysis of Variance (ANOVA) for continuous variables and Pearson's χ^2 test for categorical variables. We conducted logistic regression analysis to examine the association between immune impairment and TTV species, as well as the number of TTV species, adjusting for age, sex, and country. For multiple comparisons across TTV species, *p*-values from univariate logistic models were adjusted using the Benjamini-Hochberg procedure. Associations with a false discovery rate (FDR) < 0.1 were considered significant, in line with thresholds commonly used in high-dimensional omics studies. In addition, lifestyle-related variables such as smoking status, dietary habits, and physical activity were evaluated for potential associations with TTV viremia (using linear regression) and immune impairment (using logistic regression). As no significant associations were observed, these variables were not included as covariates in the final models. Linear regression models were also employed to explore associations between the number of TTV species and age, viral load, CMV IgG levels, inflammatory markers, and PARP-1 gene expression and PARP activity, with adjustments for age, sex, and country. Additionally, ANCOVA analysis was performed to evaluate lymphocyte subpopulations and immunological parameters in relation to TTV species with age, sex, and country as covariates. All the analyses were performed using the SPSS/Win program (version 27.0; Spss Inc., Chicago, IL). Alpha- and beta-diversity metrics were calculated using vegan R package and RStudio software (Version 2023.12.0.369 PBC, Boston, MA.). Statistical significance between different cohorts was assessed with Mann-Whitney U test for the distributions of alpha-diversity metrics. Beta-diversity was evaluated using Bray-Curtis distances on log-transformed abundance, group-level differences were tested via PERMANOVA (permutational multivariate analysis of variance). ANOSIM (Analysis of similarities) was performed to assess the strength of group separation, and beta-dispersion analysis was used to evaluate the within-group variance. Principal Component Analysis (PCA) and distance-based Redundancy Analysis (db-RDA) were performed with Bray-Curtis

dissimilarities as input. Cohort group and age were included as covariates in db-RDA model using capscale function, and statistical significance was assessed via permutation tests. Hierarchical clustering of samples and TTV species was performed using Ward.D2 linkage method. Data visualization for alpha-diversity analysis, PCA, and db-RDA plots was carried out using the ggplot R package, while the heatmap was generated using the pheatmap R package.

3 | Results

3.1 | Characteristics of the Studied Cohorts

Cohorts 1B and 1 C had a higher Charlson Comorbidity Index (CCI) compared to Cohort 1 A ($p < 0.001$). Cohorts 1B and 1 C exhibited similar characteristics, with no significant differences in blood parameters, except for a higher CRP level in Cohort 1 C (Table 1, $p < 0.05$).

3.2 | Alpha- and Beta-Diversity Analyses of TTV Species Composition

Alpha-diversity analyses (Figure S1, Supporting material) showed that cohort 1 C consistently exhibited the highest diversity values. Compared with both 1 A and 1B, cohort 1 C displayed significantly greater richness ($p = 8 \times 10^{-9}$ and $p = 1.1 \times 10^{-6}$, respectively), Shannon Entropy ($p = 1.5 \times 10^{-5}$ and $p = 3.2 \times 10^{-5}$), and Simpson diversity ($p = 4.3 \times 10^{-8}$ and $p = 1.5 \times 10^{-4}$). However, evenness did not differ significantly between 1 C and the other cohorts. Comparisons between 1 A and 1B revealed no significant differences in richness or evenness, although Shannon ($p = 0.037$) and Simpson ($p = 0.041$) indices were higher in 1B.

Beta-diversity patterns, assessed on Bray–Curtis dissimilarities of log-transformed abundance data, were consistent with these results. Principal component analysis revealed partial clustering of samples by cohort, with 1 C showing the greatest separation from the other two groups (Figure S2, Supporting material). Beta-diversity analysis are summarized on Table S1 (Supporting material). PERMANOVA confirmed significant compositional differences among cohorts (pseudo-$F = 2.962$, $p = 0.0012$), while ANOSIM indicated a significant but modest separation ($R = 0.102$, $p = 0.0002$). Nevertheless, beta-dispersion analysis revealed significant differences in within-group variance ($F = 10.709$, $p = 0.0002$), suggesting heterogeneity in community composition was also cohort-dependent.

3.3 | TTV Species Prevalence and Association With Immune Impairment

Figure 1 shows the distribution of TTV species across the three cohorts (panel A) and their relationship with age (panel B).

TABLE 1 | Characteristics of studied cohorts.

Variables	Cohort 1 A $n = 38$	Cohort 1B $n = 46$	Cohort 1 C $n = 58$	p value
Age (years)[§]	41.4 ± 4.3	68.8 ± 2.9*	69.4 ± 3.0*	< 0.001
Females %	44.7% (17)	43.4% (20)	43.1% (25)	NS
BMI	23.9 ± 2.1	25.7 ± 0.9	26.7 ± 1.2	NS
Systolic blood pressure	145.6 ± 11.6	133.5 ± 5.2	126.7 ± 6.3	NS
Diastolic blood pressure	86.2 ± 6.0	76.7 ± 2.7	76.9 ± 3.3	NS
Current smoker, % (n)	21.1% (8)	19.6% (9)	12.1% (7)	NS
CCI, median (range)^	0 (0)	1 (1-2)*	1 (1-2)*	< 0.001
WBC ($\times 10^3/\mu L$)	6.2 ± 0.5	5.8 ± 0.2	5.5 ± 0.3	NS
Neutrophils ($\times 10^3/\mu L$)	3.6 ± 0.4	3.2 ± 0.2	3.0 ± 0.2	NS
Lymphocytes ($\times 10^3/\mu L$)	1.8 ± 0.2	2.0 ± 0.1	1.8 ± 0.1	NS
Monocytes ($\times 10^3/\mu L$)	0.49 ± 0.05	0.46 ± 0.02	0.47 ± 0.03	NS
Platelets ($\times 10^3/\mu L$)	279 ± 30	224 ± 14	211 ± 17	NS
NLR	1.99 ± 0.31	1.71 ± 0.15	1.86 ± 0.18	NS
CRP (μg/L)	1.81 ± 1.36	1.19 ± 0.63	2.63 ± 0.76[§]	0.075
TC (mmol/L)	4.9 ± 0.6	5.9 ± 0.3	5.8 ± 0.3	NS
HDL (mmol/L)	1.62 ± 0.18°	1.65 ± 0.09	1.48 ± 0.10[§]	NS
LDL (mmol/L)	2.74 ± 0.49	3.57 ± 0.22	3.45 ± 0.27	NS
TG (mmol/L)	0.7 ± 0.4**	1.1 ± 0.2	1.4 ± 0.2	NS
FG (mmol/L)	5.0 ± 0.3	5.4 ± 0.1	5.3 ± 0.2	NS

Note: Data are reported as mean ± Standard Error of the Mean (SEM) or SD.[§]
Abbreviations: BMI, Body mass index; CCI, Charlson comorbidity index; CRP, C-reactive protein; FG, fasting glucose; HDL, high-density lipoprotein cholesterol; LDL, Low-density lipoprotein cholesterol; NLR, Neutrophil-to-Lymphocyte Ratio; NS, Not Significant; TG, triglycerides; TC, total cholesterol.
*$p < 0.001$ compared to Cohort 1 A; **$p < 0.001$ compared to Cohort 1 C and Cohort 1B.
°$p < 0.05$ compared to Cohort 1 A.
§$p < 0.05$ compared to Cohort 1B.

TTV1, TTV3, TTV5, TTV15, TTV20, TTV24 and TTV29 were more prevalent in Cohort 1 C than in Cohort 1B. Moreover, TTV3, TTV9, TTV15, and TTV24 were significantly less prevalent in Cohorts 1 A compared to Cohort 1 C. Additionally, TTV17 was detected in only 11 subjects from Cohort 1 C, while TTV31 were identified in 3 subjects from Cohorts 1B and 1 C. TTV species that were significantly more prevalent in older subjects compared to young adults included TTV3, TTV5, TTV9, TTV17, and TTV24. Reported p-values for differences in species prevalence across cohorts refer to univariate comparisons; Benjamini-Hochberg correction (FDR < 0.1) was applied to account for multiple testing. A significant positive association was also observed between the number of TTV species and age ($\beta = 0.368$, $p < 0.001$; Figure S3, Supporting material). No statistically significant differences were observed in TTV species prevalence between sexes (Figure S4, Supporting material).

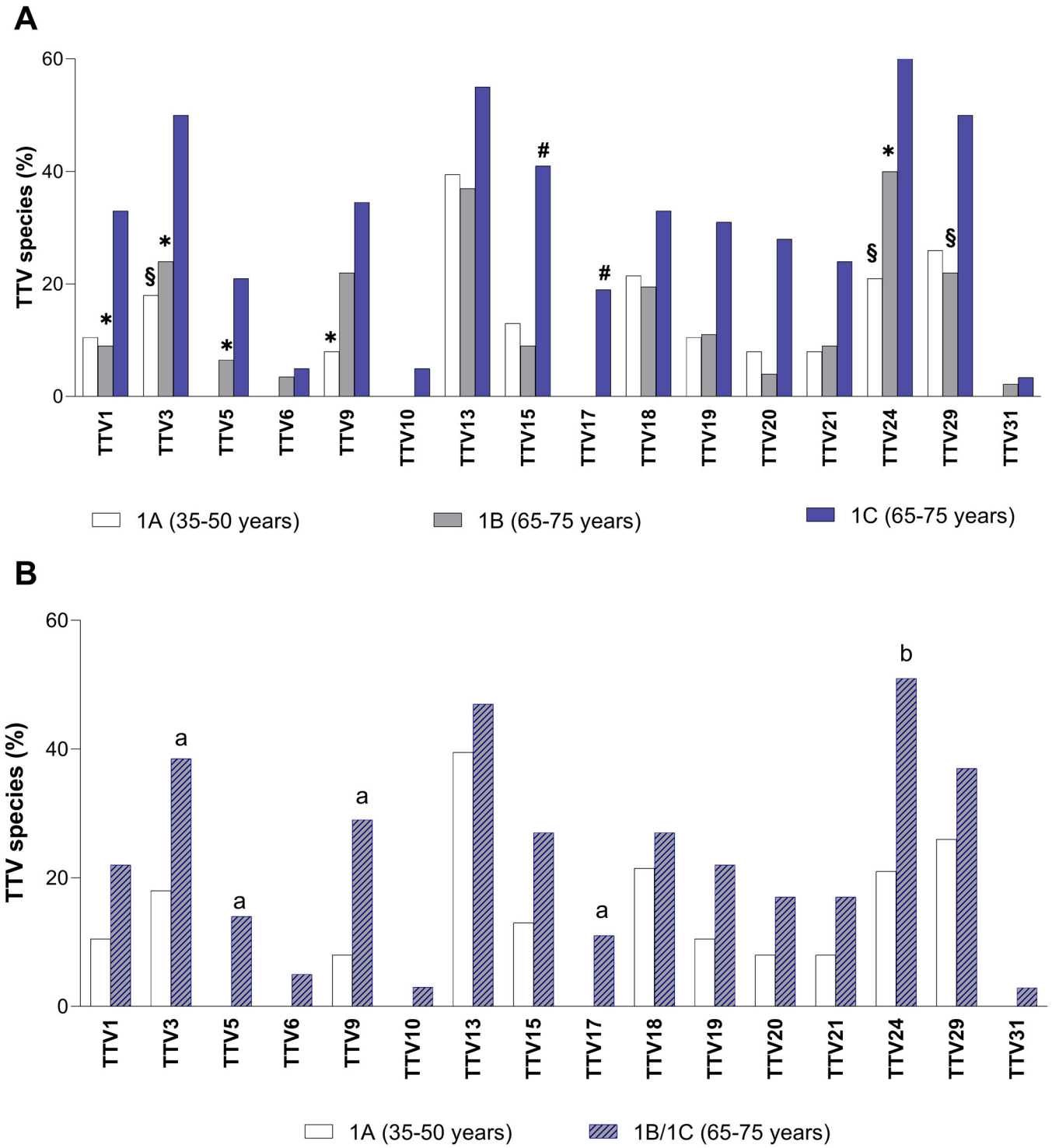

FIGURE 1 | TTV species distribution across the studied cohorts. Prevalence of TTV species across the three cohorts (A) and prevalence of TTV species by age group (B). *$p < 0.05$ compared to Cohort 1 C; $^{\S}p < 0.01$ compared to Cohort 1 C; $^{\#}p < 0.05$ compared to Cohort 1 A and 1B; $^{a}p < 0.05$ compared to Cohort 1 A; $^{b}p < 0.01$ compared to Cohort 1 A.

Table 2 shows the findings of a logistic regression analysis that investigates the relationship between TTV species and immune impairment, focusing only on older adults from cohorts 1B and 1 C. The model adjusts for age, sex, and country. A significantly increased odds ratio (OR) was observed for TTV1, TTV3, TTV5, TTV15, TTV19, TTV20, TTV21, TTV24, and TTV29. Given the multiple comparisons across TTV species, p-values from the univariate models were adjusted using the Benjamini-Hochberg procedure, with a false discovery rate (FDR) threshold of 0.1. After correction, the same species remained significantly associated with immune impairment (Table 2).

Additionally, a higher number of TTV species was linked to an increased risk of immune impairment and was positively associated with higher TTV viral load and CMV IgG levels (Figure S5, Supporting material). TTV species combinations were largely unique to each subject, with only a few coinfection profiles recurring across two or three individuals (Table S2, S3, Supporting material). To further explore community-level patterns, we performed multivariate analyses of TTV species composition using vegan R package. Distance-based redundancy analysis (db-RDA) highlighted the contribution of cohort and age in shaping the overall TTV community structure (Figure 2). Constrained axes captured a meaningful variation in TTV species diversity among samples ($F = 1.37$, $p = 0.0032$). In parallel, hierarchical clustering of TTV species abundance revealed subject-specific profiles with limited recurrence across cohorts (Figure 3), consistent with the observation that most coinfection patterns were unique to individual subjects (Table S2, S3, Supporting material). Interestingly, one cluster in the sample dendrogram was predominantly composed of subjects from cohort 1 C, and this group exhibited a greater diversity of co-infecting TTV species, confirming a cohort-specific pattern of viral richness.

3.4 | TTV Species and Lymphocyte Subpopulations

Analysis of CD4 and CD8 subpopulations, as well as their ratio, in relation to the most prevalent TTV species revealed a significant reduction in CD4 percentage in subjects positive for TTV1, TTV3, TTV9, and TTV17. Additionally, a significant increase in CD8 was observed in individuals positive for TTV3, TTV15, TTV17, and TTV20.

The CD4/CD8 ratio was significantly lower in individuals positive for TTV1, TTV3, TTV15, and TTV17 (Figure 4).

No significant changes were detected in CD4 and CD8 subsets for TTV5, TTV13, TTV18, TTV21, TTV24, and TTV29 (Figure S6, Supporting material). All differences remained significant after P-value adjustment using the Benjamini-Hochberg procedure (FDR < 0.1). Consistently, the analysis of T-cell receptor excision circles (TRECs), byproducts of T-cell receptor (TCR) gene rearrangement, showed a significant reduction in subjects positive for TTV1, TTV3, TTV15, TTV19, TTV20, and TTV24; B-cells remained unchanged regardless of viral species positivity, whereas NK-cell percentage significantly decreased in TTV5-positive subjects (Table S4, Supporting material).

TABLE 2 | Logistic regression analysis of immune impairment status in the MARK-AGE cohort.

TTV species	Immune impairment OR (95% CI)	*p* value	FDR
TTV1	5.71 (1.70−19.13)	0.005	0.0175
TTV3	3.57 (1.42−8.98)	0.007	0.0175
TTV5	4.76 (1.16−19.48)	0.030	0.052
TTV15	6.83 (2.09−22.40)	0.001	0.015
TTV19	3.34 (1.12−9.96)	0.030	0.052
TTV20	8.94 (1.79−44.45)	0.007	0.0175
TTV21	3.94 (1.13−13.69)	0.031	0.052
TTV24	3.30 (1.38−7.88)	0.007	0.0175
TTV29	3.58 (1.42−8.97)	0.006	0.0175
N. TTV species	2.63 (1.45−4.77)	< 0.001	—

Note: The model was adjusted for age, sex and country.
Abbreviation: FDR, False discovery rate.

3.5 | Association of TTV Species With PARP-1 Gene Expression and PARP Activity

PARP-1 gene expression was measured in a subgroup of subjects distributed as follows: 31 from Cohort 1 A, (mean age: 42.1 ± 4.3 years; 16 females, 15 males), 41 from Cohort 1B (mean age: 68.9 ± 2.8 years; 18 females, 23 males), and 40 subjects from Cohort 1 C (mean age: 69.2 ± 2.9 years; 18 females, 22 males). A positive association with the number of TTV species was observed ($\beta = 0.243$, $p < 0.05$; Figure 5A).

PARP-1 activity was analyzed in a subgroup of 74 subjects, distributed as follows: 24 from Cohort 1 A (mean age: 42.0 ± 4.2 years; 11 females, 13 males), 30 from Cohort 1B (mean age: 69.9 ± 3.1 years; 15 females, 15 males), and 20 from Cohort 1 C (mean age: 70.2 ± 3.1 years; 10 females, 10 males). A positive association with the number of TTV species was found ($\beta = 0.327$, $p < 0.01$; Figure 5B).

Moreover, we investigated whether PARP-1 gene expression and PARP activity were modulated in relation to specific TTV species. TTV9 and TTV20 were associated with increased PARP-1 gene expression and PARP activity (Figure 6), and these associations remained significant after *P*-value adjustment using the Benjamini-Hochberg procedure (FDR < 0.1), while no significant associations were observed for the other species (data not shown).

3.6 | Association of TTV Viremia and Species With Inflammatory Parameters

The potential association between the number of TTV species and TTV viremia with systemic inflammation markers, including ceruloplasmin, homocysteine, NLR, ferritin, albumin, CRP, and α2-macroglobulin, was evaluated using linear regression analysis. The findings revealed that the number of TTV species did not exhibit a significant correlation with these

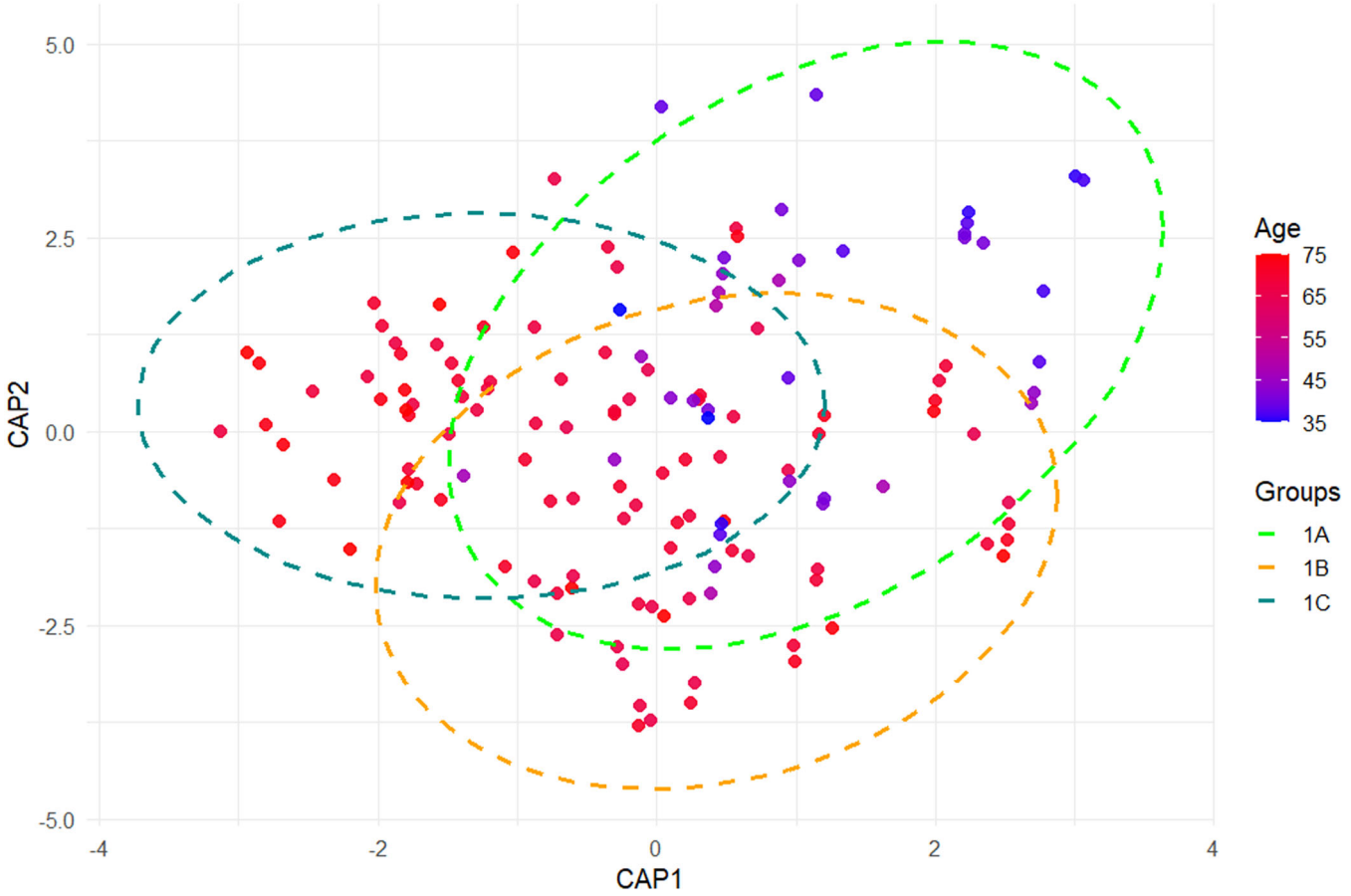

FIGURE 2 | Distance-based Redundancy Analysis (db-RDA) of TTV species composition. Ordination plot based on Bray-Curtis distance matrix of log-transformed viral abundance data. The db-RDA model includes cohort group and age. Sample points are colored according to age (blue to red gradient), while 95% confidence ellipses are shown for each cohort (1 A, 1B, and 1 C are colored in green, orange and dark cyan, respectively).

parameters. However, TTV viral load demonstrated a positive association with CRP and α2-macroglobulin ($\beta = 0.254$, $p = 0.014$ and $\beta = 0.316$, $p = 0.003$, respectively; see Table S5, Supporting material). Additionally, the potential variation in CRP and α2-macroglobulin levels based on different TTV species was investigated. It was found that only TTV15-positive subjects exhibited elevated α2-macroglobulin levels compared to TTV15-negative individuals, while CRP levels remained unaffected. No significant differences were noted for the other TTV species (Table S6, Supporting material).

4 | Discussion

TTV viremia has been widely recognized as a biomarker of immune suppression in various clinical settings [1, 4, 5, 8, 39]. However, the role of genetic TTV variability in immunosenescence and age-related immune status remains unexplored. This study indicates that the genetic complexity of TTV, measured as number of different TTV species present in a single individual, is significantly higher in older adults with immune impairment, suggesting that increased TTV species diversity may signify a progressively dysfunctional immune system.

This observation is consistent with previous studies that report higher TTV loads correlating with age and immune suppression [1, 8]. Additionally, certain TTV species, such as TTV3, TTV5, TTV9, and TTV24, have been more frequently observed in immunocompromised populations [36, 40, 41], while others like TTV1 and TTV2 have been linked to immunosuppression, especially in those with chronic viral infections or autoimmune disorders [42].

Several studies have reported that the most common TTV species in kidney transplant recipients include TTV3, TTV29, TTV24, and TTV22 [41, 43]. Reyes et al. (2024) [36] identified TTV3 (63.3%) and TTV24 (53.3%) as the most prevalent species in renal transplant patients, followed by TTV9 and TTV13 (43.3%). In the post-transplant period, the predominant species were TTV3 (55.6%), followed by TTV24 (44.4%) and TTV5 (38.9%) [36]. Studies involving patients with HCV, HBV, or HIV infection noted TTV species 13 and 24 as the most prevalent, followed by TTV1 and 5 [40, 43]. Our findings also indicate that TTV17, though well-represented in our results, has not been highlighted in other studies concerning prevalence. A recent study suggested that TTV7 was present at high viral loads in patients with Kawasaki disease [37].

In the context of aging, it remains unclear whether the expansion of different TTV species observed during immune dysregulation is primarily driven by new infections or by reactivation of latent strains already present within the host. Given the high prevalence and persistent nature of TTV, it is plausible that

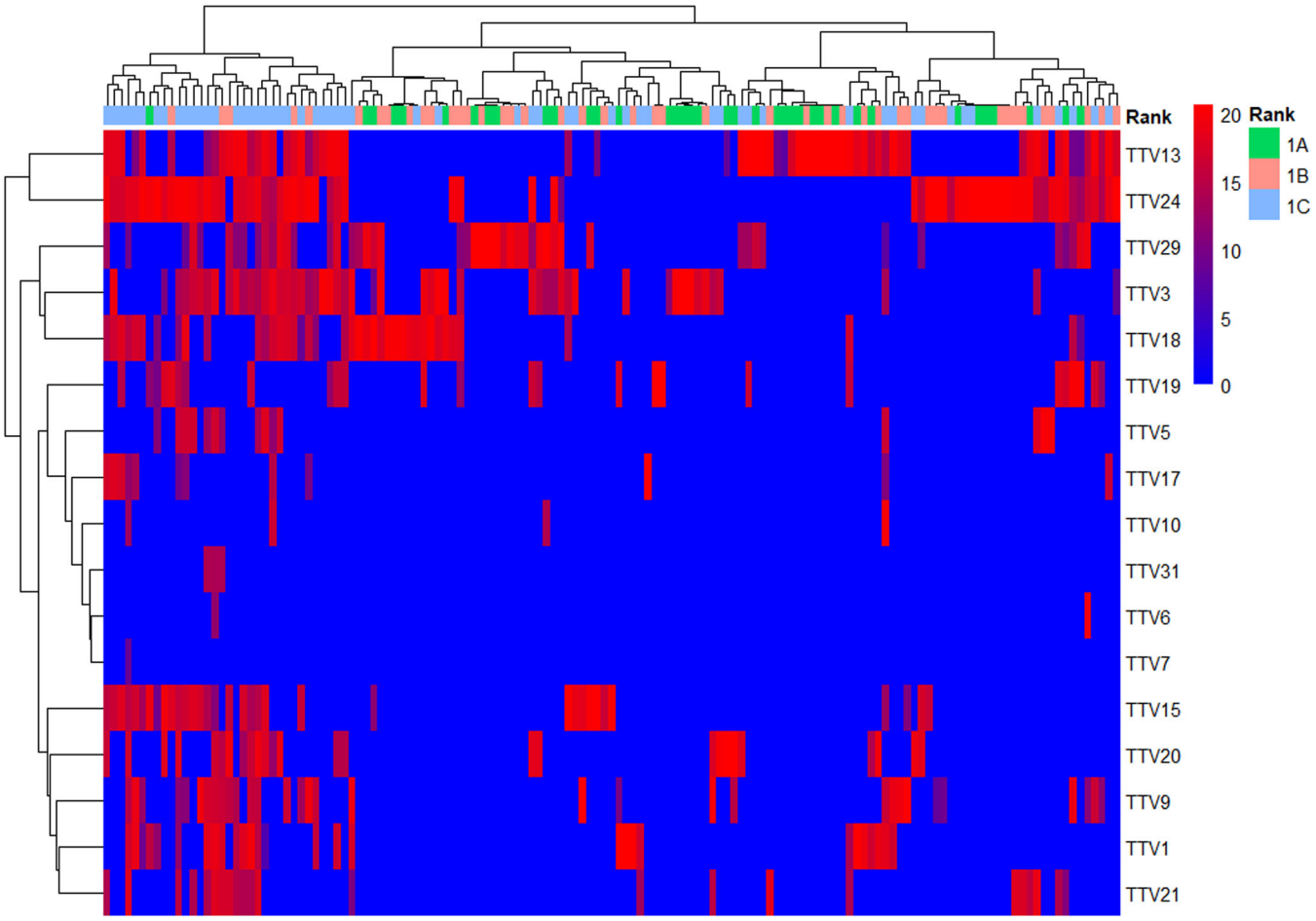

FIGURE 3 | Heatmap of TTV species abundance across cohort groups. Hierarchical clustering of log-transformed TTV species abundance across individual samples. Rows represent TTV species, columns represent subjects annotated by cohort group (1 A, 1B, and 1 C are colored in green, orange and dark cyan, respectively). Clustering was performed using Ward.D2 linkage on both axes. The color gradient (blue to red) reflects relative abundance levels.

immunosenescence facilitates the reactivation and outgrowth of pre-existing, low-abundance species that were previously controlled by immune surveillance. However, exposure to new TTV variants through external sources cannot be excluded, especially in older individuals with weakened mucosal and systemic immunity. Longitudinal studies with deep sequencing are needed to disentangle reactivation from reinfection dynamics in aging population. The increased diversity of TTV species, observed during immune dysregulation may reflect the lack of selective immune pressure, which under normal conditions constrains less dominant variants. In immunosenescent individuals, the loss of this control may allow expansion of multiple co-existing strains, including those with higher replication capacity, immune evasion properties, or specific tropism. This could explain the greater prevalence of species such as TTV1, TTV3, TTV9, TTV13, TTV15, TTV24 and TTV29 in subjects with high TTV load and immune dysfunction. This aligns with recent metagenomic evidence that immune dysfunction favors mixed anellovirus infections [44]. Distance-based Redundancy Analysis confirmed that age and cohort contributed to shaping overall TTV community structure, while clustering underscored the largely individualized nature of species profiles, supporting the view that TTV diversification in aging reflects both the loss of selective immune control and the emergence of individualized viral communities. Another interesting finding of the study is the association between TTV species diversity and lymphocyte homeostasis. Specifically, individuals harboring certain TTV species, including TTV1, TTV3, TTV15, and TTV17, exhibited a significantly reduced CD4/CD8 ratio, a known marker of immunosenescence. The presence of multiple TTV species was linked to lower levels of TRECs, indicating reduced thymic output and naive T-cell production. Since thymic involution is a hallmark of aging and contributes to immune dysfunction [22], our results suggest that specific TTV species may preferentially persist in individuals with compromised T-cell renewal. Supporting this, previous evidence has linked TTV viremia with CD4 and CD8 counts, as well as the CD4/CD8 ratio, in young individuals with vertically acquired HIV [45].

In this context, TTV elicits a universally detectable TTV-specific CD8 + T-cell response, however, unlike HCMV, its replication is less efficiently controlled by virus-specific CD8 + T cells [46]. Moreover, specific TTV peptides have been shown to inhibit the activation of NKG2A + CD8 + T and NKG2A + NK cells, contributing to persistent viremia [46]. Consistent with this immunomodulatory effect, we observed fewer NK cells in

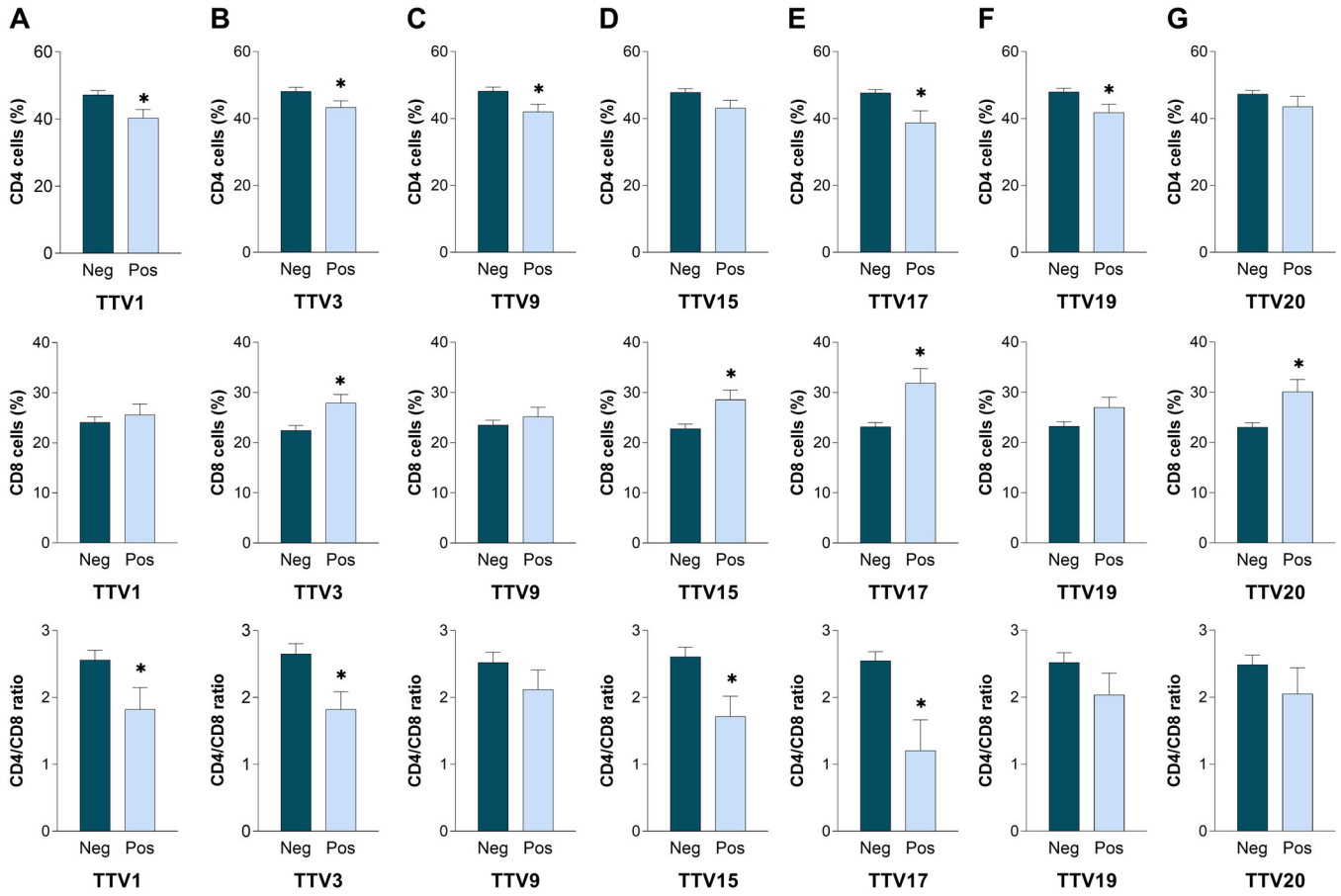

FIGURE 4 | CD4 and CD8 subsets according to TTV species. Reduced CD4 percentages were observed in subjects positive for TTV1, TTV3, TTV9, and TTV17, while increased CD8 percentages were detected in subjects positive for TTV3, TTV15, TTV17, and TTV20. The CD4/CD8 ratio was significantly lower in subjects positive for TTV1, TTV3, TTV15, and TTV17. *$p < 0.05$ compared to TTV negative subjects. p-values from univariate models were adjusted using the Benjamini-Hochberg procedure (FDR < 0.1).

TTV5-positive subjects, suggesting that this TTV species may influence NK cell dynamics.

Beyond immune cell alterations, our study also explored the potential link between TTV and systemic inflammation, a key component of "inflammaging" [22]. Although the number of TTV species alone was not significantly associated with inflammatory markers, the overall TTV load positively correlated with CRP and α2-macroglobulin levels, suggesting that high TTV replication may reflect an inflammatory state. Our previous studies have reported increased inflammation in older adults with ischemic heart disease and high TTV viral load [3], as well as an association between TTV and IL-8 levels in an elderly cohort, with TTV miRNA-t3b positively correlating with CRP and IL-6 [1]. Moreover, some evidence demonstrated increased TTV viremia in inflammatory conditions such as COVID-19 and chronic viral infections [17, 47]. Interestingly, among individual TTV species, only TTV15 was significantly associated with α2-macroglobulin elevation, implying a pronounced relationship with inflammatory processes. However, the directionality of this association remains unclear; it is possible that inflammation facilitates TTV replication rather than TTV directly inducing an inflammatory response. Future studies should aim to elucidate the mechanistic basis of this relationship.

A novel aspect of our study is the strong link between TTV species diversity and PARP-1 activation. PARP-1 is a key regulator of cellular homeostasis, DNA repair, and inflammatory signaling [48] and its overactivation has been implicated in aging and age-related diseases [49, 50]. PARP-1, through ADP-ribosylation of viral proteins, plays a crucial role in the antiviral response [51, 52] and might also be involved in TTV replication. We found that individuals harboring a greater number of TTV species exhibited increased PARP-1 gene expression and enzymatic activity, particularly in TTV9- and TTV20-positive subjects. These findings suggest that certain TTV species may contribute to cellular stress responses, either directly through viral interactions with host pathways, or indirectly by persisting in individuals with increased oxidative stress and DNA damage. Supporting this, we recently reported a correlation between higher TTV viremia and increased DNA damage in older COPD patients [53]. However, our cross-sectional design does not allow us to determine whether PARP-1 activation is a cause or consequence of TTV persistence. While it is possible that chronic immune activation and oxidative stress in aging individuals lead to increased PARP-1 expression, which may in turn impair immune surveillance and promote TTV expansion, the reverse scenario is also conceivable. TTV infection or replication itself might contribute to cellular stress, DNA damage signaling, and sustained PARP-1 activation. Disentangling these

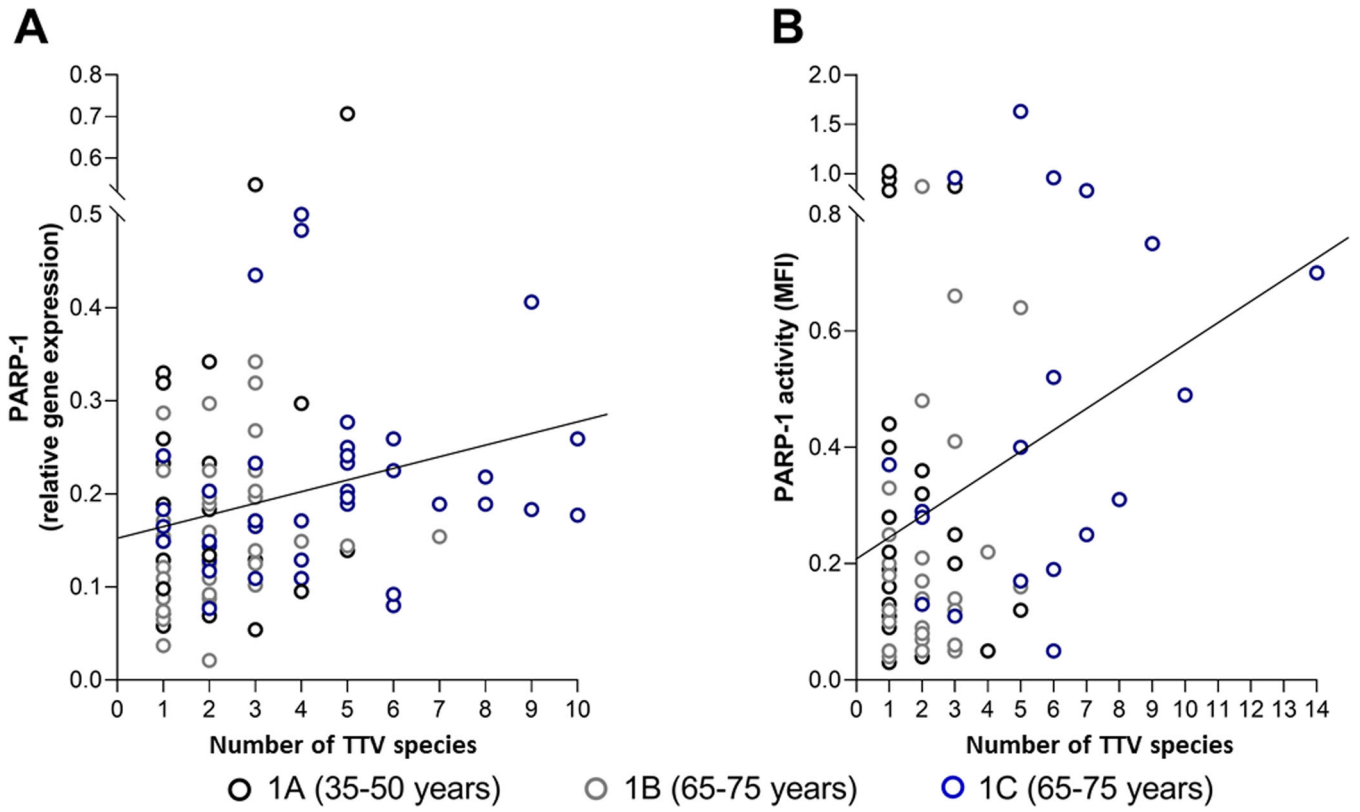

FIGURE 5 | Scatter plots and linear regression between number of TTV species and PARP-1 gene expression/PARP activity in the studied cohorts. A significant positive association was observed between the number of TTV species and PARP-1 gene expression ($\beta = 0.243$, $p < 0.05$; $N = 112$) (A) as well as PARP activity ($\beta = 0.327$, $p < 0.01$) (B) in the samples analyzed. Analysis adjusted for age sex and country.

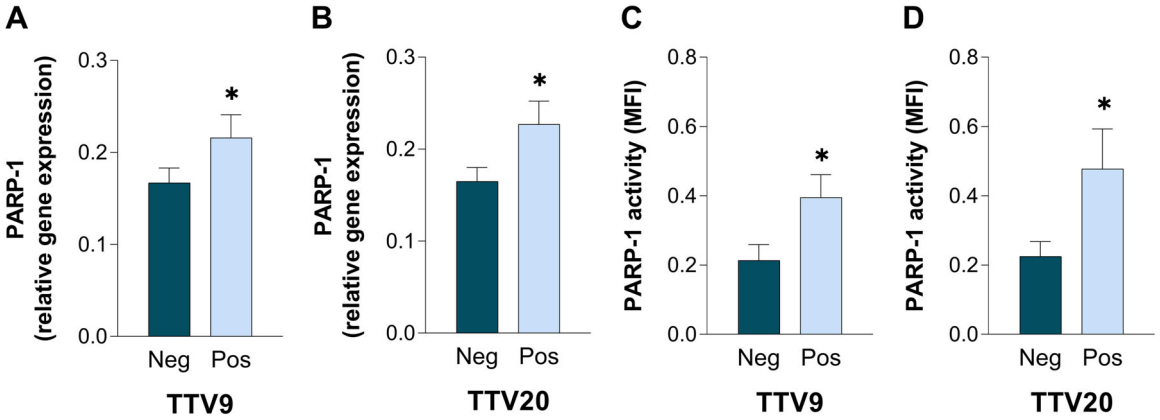

FIGURE 6 | Association of PARP-1 gene expression and PARP activity with TTV9 and TTV20 species. In subjects positive for TTV9 and TTV20, PARP-1 gene expression (A, B) and PARP-1 activity (C, D) were significantly higher compared to negative subjects. *$p < 0.05$, ANCOVA analysis adjusting for age, sex and country. P-values from univariate models were adjusted using the Benjamini-Hochberg procedure (FDR < 0.1).

interactions will require dedicated longitudinal studies, ideally integrating molecular virology with host response profiling in relevant populations.

From an immunological perspective, PARP-1 acts as a coactivator of the NF-κB transcription factor, promoting the expression of pro-inflammatory genes [52]. It also regulates both adaptive and innate immunity, including the suppressive function of regulatory T cells (Tregs) [54, 55]. Given the emerging role of PARP-1 in immunosenescence and inflammatory age-related diseases [56], further investigation into the relationship between TTV and PARP-1 activation is warranted.

Despite these compelling findings, our study has several limitations. First, the cross-sectional design prevents establishing causal relationships between TTV diversity, immune impairment, and inflammation. It remains unclear whether increased TTV diversity is a consequence of immunosenescence or contributes to its progression. Longitudinal studies are needed to

disentangle these relationships and better understand the dynamics of TTV evolution in aging individuals.

Second, while our sample size was sufficient to detect major associations, subgroup analyses (*e.g.*, examining individual TTV species) were limited by low statistical power. Larger studies are necessary to confirm our findings and explore potential confounding factors such as medication use, comorbidities, and lifestyle variables. However, in our cohort, data on smoking, dietary habits, and physical activity were available and tested in sensitivity analyses (Tables S7, S8, Supporting material). These variables were not significantly associated with either TTV viremia or immune impairment, and were not included as covariates in the main models. Third, our study relied on NGS to characterize TTV species diversity, but this approach is unable to assess their transcriptional activity or replication competence. The detection of viral DNA alone does not necessarily indicate active infection. Future research should integrate transcriptomic and proteomic approaches to determine which TTV species are transcriptionally active and capable of replication in vivo, as well as to better characterize their potential immunomodulatory roles and interactions with host pathways. Integrating transcriptomic and proteomic analyses would allow for a more comprehensive understanding of TTV′s biological impact.

In conclusion, our study demonstrates that TTV species diversity is strongly associated with markers of immunosenescence, systemic inflammation, and PARP-1 activation in older adults. These findings suggest that TTV could serve as a valuable biomarker of immune aging, complementing existing markers such as CMV serostatus and CD4/CD8 ratio. However, further research is needed to clarify the mechanistic underpinnings of these associations and to evaluate the clinical relevance of TTV monitoring in aging populations. If confirmed, TTV species profiling could be integrated into routine immunological assessments, offering a novel approach to tracking immune health and predicting age-related disease risk.

Author Contributions

Federica Novazzi: writing review and editing, formal analysis, project administration, **Pietro Giorgio Spezia:** writing review and editing, formal analysis, **Francesca Drago Ferrante:** formal analysis, **Angelo Paolo Genoni:** formal analysis, **Paolo Antonio Grossi:** writing review and editing, supervision, **Nicasio Mancini:** writing review and editing, supervision, **Marco Malavolta:** writing review and editing, supervision, **Marta Balietti:** writing review and editing, **Francesco Piacenza:** writing review and editing, software, **Serena Marcozzi:** writing review and editing, **Carlo Fortunato:** writing review and editing, methodology, **Gretta Veronica Badillo Pazmay:** writing review and editing, methodology, **Laura Cianfruglia:** writing review and editing, methodology, **Alexander Bürkle:** writing review and editing, project administration, funding acquisition, **María Moreno-Villanueva:** writing review and editing, methodology, **Martijn E.T. Dollé:** formal analysis, data curation, **Eugène Jansen and Tilman Grune:** resources, biobank, **Efstathios S. Gonos:** resources, formal analysis, **Claudio Franceschi:** resources, formal analysis, **Miriam Capri:** resources, formal analysis, **Michele Zampieri:** writing review and editing, **Paola Caiafa:** formal analysis, **Fabio Ciccarone:** methodology, **Anna Reale:** methodology, **Birgit Weinberger:** resources, **Ewa Sikora:** resources, **Florence Debacq-Chainiaux:** resources, **Wolfgang Stuetz:** formal analysis, **Mikko Hurme:** resources, **P. Eline Slagboom:** formal analysis, **Jürgen Bernhardt:** resources, **Olivieri Fabiola:** writing review and editing, supervision, **Fabio Filippini:** formal analysis, **Fabrizio Maggi:** writing review and editing, supervision, conceptualization, **Robertina Giacconi:** writing review and editing, conceptualization, project administration, funding acquisition.

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Ethics Statement

All information was accessed in accordance with the applicable laws and ethical requirements for the study period concerned and was compliant with the Declaration of Helsinki. The Local Research Ethics Committees of the respective recruiting centers provided ethical approval for the MARK-AGE project, which was registered retrospectively at the German Clinical Trials Register (DRKS00007713; Ethics Committee No.: 2008 075-f, Ethik-Kommission bei der Landesärztekammer Baden-Württemberg).

Consent

Informed consent was obtained from all subjects involved in the study. The VIROMA study was approved by the Ethics Committee of IRCCS INRCA (No. CE INRCA 23011).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request. The data of this study are available on request from the authors.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.
Supplementary material novazzi et al. **Table S1.** Multivariate tests of beta diversity differences among groups. **Table S2.** Read counts and relative abundance of TTV species in individual subjects. **Table S3.** Cohort distribution of TTV single-species infections and coinfections.