## Liver resection with thrombectomy as a treatment of hepatocellular carcinoma with major vascular invasion: results from a retrospective

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**Torquetenovirus (TTV) load is associated with mortality in Italian elderly subjects**

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**Abstract**

An age-related dysregulation of immune response, known as immunosenescence, contributes to increased susceptibility to infections, frailty and high risk of mortality in the elderly. Torquetenovirus (TTV), a circular, single-stranded DNA virus, is highly prevalent in the general population and it may persist in the organism, also in association with other viruses such as Cytomegalovirus (CMV), causing chronic viremia. The relationship that TTV establishes with the immune system of infected hosts is not clear. It is known that TTV encodes microRNAs (miRNAs) that might contribute to immune evasion and that the highest viral loads are found in peripheral blood cells. Moreover, it is suspected that TTV infection lead to increased production of inflammatory mediators, thus playing a role in immunosenescence. We investigated the association of TTV load and miRNAs expression with inflammatory and immune markers and the influence of TTV load on mortality within a cohort of 379 elderly subjects who were followed up for 3 years. TTV DNA load in polymorphonuclear leukocytes was slightly positively correlated with age and negatively associated with serum albumin levels and NK cell activity. A marginal positive correlation between TTV DNA load, monocytes and IL-8 plasma levels was found in females and males respectively. TTV DNA copies ≥ 4.0 log represented a strong predictor of mortality (Hazard ratio = 4.78, 95% CI: 1.70-13.44, after adjusting for age, sex and the main predictors of mortality rate) and this association remained significant even after the CMV IgG antibody titer was included in the model (HR = 9.83; 95% CI: 2.48-38.97; N=343 subjects). Moreover, multiple linear regression model showed that TTV miRNA-t3b of genogroup 3 was inversely associated with triglycerides, monocytes and C-reactive protein, and directly associated with IL6. Overall these findings suggest a role of TTV in immunesenescence and in the prediction of all-cause mortality risk in Italian elderly subjects. Further studies are needed to fully understand the pathogenic mechanisms of TTV infection during aging.

Keywords: TTV, aging, immunesenescence, mortality, NK cell activity

## 1. Introduction

Aging is associated with a variety of physiological changes, including a decline in immune function known as "immunosenescence." This condition is characterized by age-related dysfunction of the immune system, affecting both innate and adaptive immunity, causing a reduced response to pathogens and to vaccines with greater risk of frailty and mortality in elderly subjects (Pawelec et al.,2014). NK cells are involved in the elimination of viral-infected cells as well as in the innate and adaptive immunological regulation through the production of cytokines and chemokines (Camous et al.,2012). During aging, NK cell dysfunction may contribute to the imbalance between inflammatory and anti-inflammatory signals contributing to a state defined as "inflammaging (Ventura et al., 2017). The measure of Cu/Zn ratio may represent a sensitive biomarker of systemic inflammation and "inflammaging" status, because it is strongly related to circulating inflammatory biomarkers in old population (Malavolta et al., 2010; Giacconi et al., 2017).

Several herpesviruses, such as cytomegalovirus (CMV), cause chronic antigen stimulation, evade NK cell recognition (Charpak-Amikam et al., 2017) and have been associated with immunosenescence and telomere length attrition (Nikolich-Žugich and van Lier,2017; Stowe et al., 2012). Some evidence shows that CMV seropositivity, associated with higher C-reactive protein, increased the risk for all-cause mortality and cardiovascular diseases (CVD)-related mortality (Simanek et al., 2011), while the higher anti-CMV titres increased the risk of death in elderly over 80 years (Matheï et al., 2015). Currently there are no simple biomarkers for the overall level of immune function decline conferred on a patient with the age. Thus, new biomarkers are needed to accurately assess the degree of immunosenescence in elderly people, and to help physicians in the management of aged patients. Recently, Torquetenovirus (TTV) viremia has been proposed as a potentially simple and general measure of immune system function of infected host. TTV, originally isolated from a patient suffering from posttransfusion hepatitis (Nishizawa et al., 1997), is the prototype of a vast group of viruses with a small, circular single-stranded DNA virus belonging to Anelloviridae family and it is the most representative and abundant virus of the human virome (Focosi et al., 2016).

Since TTV was first detected in blood samples, it was referred to as transfusion-transmitted (TT) virus (Nishizawa et al., 1997), but other ways of TTV transmission including parenteral, oro-fecal, respiratory, sexual and vertical transmission have been reported (Haloschan et al., 2014). Due to its high prevalence (70-90 %) in the general population, a causative role

of TTV in human diseases remains uncertain. However, a higher prevalence of TTV was found to be associated with several pathologies such as viral hepatitis, respiratory diseases, sepsis, enteritis, and cancer (Rosa et al., 2017; Feyzioğlu et al, 2014; Walton et al., 2014; Brassard et al., 2016; Szládek et al., 2005). In addition, certain TTV species/genotypes were suggested to be more pathogenic than others in patients with liver disease (Tokita et al., 2001). More recently, it has been shown that TTV encodes microRNAs (miRNAs) (Kincaid et al., 2013; Vignolini et al., 2016) and that some of these miRNAs may interfere with cytokine signalling promoting virus persistence and immune evasion (Kincaid et al., 2013).

However, the possible involvement of TTV and its miRNAs in immunesenescence or increased risk of mortality in elderly people is currently unknown. The purpose of this study was to investigate the association of TTV load and miRNAs with age, inflammatory and immune markers as well as the risk for all-cause mortality in elderly population after three-year follow-up.

## 2. Materials and methods

### 2.1. Subject population

The study included n. 379 noninstitutionalised elderly subjects (age range 60–105 years) born and living in east central Italy (the Marche Region) recruited in the years 2007-2008. The exclusion criteria included autoimmune diseases, neurodegenerative diseases, infections, cancer, Crohn's disease and acrodermatitis enteropathica, kidney disease, liver disease, sickle cell anaemia, chronic skin ulcerations and endocrine disorders. Subjects also had to be free of anticonvulsants, anti-depressives drug and antibiotics. A standardised questionnaire was administered to collect data regarding the medical history, anti-inflammatory medication (acetylsalicylic acid, corticosteroids and non-steroidal anti-inflammatory drugs (NSAIDs), statins, ACEinhibitors and fibrates) and personal habits. To ensure validity, the family doctor was requested to check the completed questionnaire. Subjects with CVD (angina, arrhythmia, compensated heart failure) were included in the study if these conditions did not compromise the individual's independence. The healthy status was evaluated by a specific questionnaire on health and morbidity planned for the study and filled in by the general practitioner after medical examination and analysis of the clinical record. Hypertension was defined as seated systolic blood pressure C160 mmHg, diastolic pressure C95 mmHg or both, or self-reported hypertension and if the subject was already taking anti-hypertensive medication. The cohort was followed up for 3 years using

mortality records held by the General Register Office in the subject's place of residence. Death certificates were examined by a medical doctor to determine the main cause of death. All subjects signed an informed consent, which was approved by the Ethical Committee of INRCA. Biological samples were collected in the INRCA biobank and made anonymous.

## 2.2. Laboratory determinations

Venous peripheral blood samples, collected after an overnight fast, underwent basal biochemical laboratory determinations. Serum, plasma, and polymorphonuclear leukocytes, were separated, aliquoted and stored frozen at −80 °C in the Biological Bank of INRCA until analysis. The blood haematocrit and haemoglobin concentration, were determined by automated blood analysers. Serum total cholesterol, HDL-cholesterol, triglycerides, were measured using an automatic analyser under conventional enzymatic methodology. Glucose, creatinine and albumin levels were determined using standard laboratory methods. C-reactive protein (CRP) levels were detected by CardioPhase hsCRP assay (Dade Behring Inc Deerfield, IL). Immunoglobulin G levels of CMV-specific antibodies were determined in plasma samples by the Liaison assay (LIAISON® CMV IgG II; Diasorin) according to the manufacturer's specifications and using the Liaison analyzer (DiaSorin, Italy).

## 2.3. Polymorphonuclear leukocytes (PMNLs) sample preparation

PMNLs were separated by sedimentation of 3−7 mL of heparinized blood sample in a 6% dextran solution (5 vol. blood/1 vol. dextran solution). Leukocytes were centrifuged at 2500 rev/min for 10 min at room temperature, and the resulting pellet was washed in 0.8% NH4Cl for 1−2 min to eliminate red blood cell contamination. PMNLs were then centrifuged and resuspended in 1 mL of phosphate-buffered saline (PBS), pH 7.4. PMNLs aliquots were stored at −80 ∘C until DNA extraction.

## 2.4. TTV DNA detection and quantification

TTV load was assessed in PMNLs where it has been reported that the viral load is highest (Takahashi et al., 2002). Viral DNA was extracted from blood PMNLs using The Wizard® Genomic DNA Purification Kit (Promega Italia s.r.l., Milan, Italy), as specified by the manufacturer. Presence and load of TTV DNA were determined in a single step TaqMan PCR assay as described elsewhere (Maggi et al., 2005). This assay uses primers designed on a highly conserved segment of the untranslated region of the viral genome and has therefore the capacity to detect all the species in which TTV is actually classified. TTV loads were expressed as the number of viral DNA copies per μg of genomic DNA extracted by PMNLs. The lower limit of detection was 10 copies of TTV DNA per μg genomic DNA. The

procedures used to quantitate the copy numbers and assess specificity, sensitivity, intra- and inter-assay precision, and reproducibility have been previously described (Maggi et al., 2005). In a limited group of patients, viral DNA was also extracted from 200 µl plasma samples using QIAamp DNA minikit® (Qiagen, Chatsworth, CA, USA), and amplified as described above. The lower limit of sensitivity was 10 TTV genomes per ml of plasma.

## 2.5. TTV miRNAs detection and quantification

Total RNA was isolated from plasma exosomes obtained from 250 µl plasma processed with Plasma/Serum Circulating and Exosomal RNA Purification kit (Norgen Biotek Corp., ON, Canada) and following the manufacturer's protocol. miRNA expression was analysed and quantified with a commercial quantitative RT-PCR miRNA assay (Life Technologies, Foster City, CA) and using primers targeting the miRNA mature region previously described (Vignolini et al., 2016). In particular, TTV miRNAs t1a of genogroup 1, t3b of genogroup 3, and tth8 of genogroup 5 were selected according to predictive computational analysis (Kincaid et al., 2013) and because found to be the most prevalent in plasma samples of patients previously studied (16Vignolini et al 2016). Each reaction was carried out in triplicate with 10 ng RNA and included negative controls (no template) and $10^1$–$10^6$ copies of positive control (synthetic oligonucleotide template). The lower limit of detection was 10 copies of TTV miRNA per ng RNA. As assessed by several reactions performed in preliminary experiments and various conditions, the assay proved specific and reproducible. Preliminarily each TTV miRNA oligonucleotide standard was tested without amplification of unrelated target and with <0.5Ct value interassay variation.

## 2.6. Zinc and copper plasma level determinations

The plasma zinc (Zn) and copper (Cu), were determined by a Thermo XII Series ICP-MS (Thermo Electron Corporation, Waltham, MA, USA) by adapting methods used for the measurement of trace elements in human plasma with slight modifications (Malavolta et al., 2010). Plasma samples were diluted 1:10, with a diluent containing 0.1% triton and 0.15% HNO3, to ensure that trace elements are maintained in solution and to favour the washout of these elements between samples. External calibration solutions containing Zn, and Cu (blank to 2000 ppb) were prepared by serial dilution of a parent multi-element solution (1000 ppm for Zn and Cu) (VHG Labs, Manchester, USA), using the same diluent used for the samples. Rhodium (Rh), at 200 ng/ml was used as internal standard. Data were acquired for 66Zn, 65Cu.

Quality of the analysis was assured by assessment of "quality standard samples" (SERONORM™ TRACE ELEMENT SERUM, Sero AS, Billingstad, Norway). Zn and Cu

levels of the quality standard samples were within 10% of the certified levels, as previously reported (Malavolta et al., 2010). The instrument was operated with a Peltier cooled impact bead spray chamber, single piece quartz torch (1.5 mm i.d. injector) together with Xi interface cones and a Cetac-ASX 100 autosampler (CETAC Technologies, Omaha, NE). A Burgener Trace nebulizer was used as this device does not block during aspiration of clinical samples. The instrument was operated in standard mode (non-CCT), using 1400 WRF power, 1.10 L min−1 nebulizer gas flow, 0.70 L min−1 auxiliary gas flow, 13.0 L min−1 cool gas flow, 70 ms dwell time, 30 s sample uptake and 35 s wash time (2 repeats per sample).

## 2.7. NK cell lytic activity

K562 tumor target cells (1 x 10$^6$) were labelled with 15 µM calcein-AM (Molecular Probe, Eugene, OR) for 30 min at 37 °C and washed 2 times with cold complete medium. Labelled target cells (5 x10$^3$/50 µl ) and varying numbers of cryopreserved and thawed effector cells (from 5 x 10$^6$ to 5 x 10$^3$ PBMC/100 µl) were incubated in triplicate in V-bottom 96-well plates (Effector/Target E/T ratios from 50/1 to 1/1). After incubation at 37 °C in 5% CO2 for 4 h, 75 µl of each supernatant were harvested, transferred into new plates and measured by a dual-scanning microplate spectrofluorimeter (Spectramax Gemini – Molecular Devices, Sunnyvale, CA) (Neri et al., 2001).

Data were expressed as arbitrary fluorescent units (AFU). Percent lytic activity was calculated according to the formula [(test AFU - spontaneous AFU)/(maximum AFU - spontaneous AFU)] x 100. Spontaneous AFU represents Calcein AM released from target cells in medium alone and maximum AFU is Calcein AM released from target cells lysed in medium plus 2% Triton X-100, each measured in at least six replicate wells.

## 2.8. Multiple immunoassay for chemokine and cytokine plasma assessment

Fourfold diluted plasma samples were assayed in duplicate using commercially available multiplex bead-based immunoassay kits. IL-6 and IL-8 concentrations were simultaneously evaluated using multiplex reagent kits (Bio-Rad Laboratories, USA). Values presenting a coefficient of variation beyond 10% were discarded before the final data analysis. Data were analysed using the Bio-Plex Manager software version 6.0 (Bio-Rad Laboratories, USA). Values were expressed as pg/ml.

## 2.9. Telomere length analysis

Telomere length was determined in peripheral blood mononuclear cells (PBMC) by flow-FISH methodology by using the Telomere PNA kit/FITC from Dako (Glostrup, Denmark) according to the manufacturer's instructions. Samples were then analyzed by flow cytometry

(Coulter Epics XL, Coulter, USA) and relative telomere length was determined by comparing isolated test cells with a control cell line (Human T-cell leukaemia 1301 cell line).

## 3. Statistical analysis

Pearson Correlation between TTV DNA loads (log-transformed data) and age was investigated stratifying by gender. A linear regression model for determining the associations between TTV DNA loads and albumin, NK cell activity, monocytes percentage and IL-8 plasma levels was performed. Log transformation of the variables was carried out if they were not normally distributed as assessed by the Kolmogorov-Smirnov test. The relationship between TTV DNA loads and all-cause and CV mortality was investigated by Kaplan–Meier analysis and by Cox regression analyses. The predictive model included age, sex, Cu/Zn, CRP, triglycerides, total cholesterol, albumin and glucose serum  levels as potential covariates.

The automatic linear modelling procedures implemented in SPSS were carried out to explore the main predictors of TTV DNA loads or miRNAs following a procedure previously described (Giacconi et al., 2017). The variables inserted were: age, gender, TTV DNA loads or miRNAs, C-reactive protein, albumin, Cu/Zn ratio, erythrocytes count, hemoglobin, leucocyte count, neutrophils, monocytes, lymphocytes percentages, BMI, lipid profile, NK cell activity, IL-6 and IL8. Main predictors of TTV DNA loads or miRNA were also confirmed using linear regression models. All statistical analyses were performed with SPSS Statistics version 22.0.0 (IBM, IL, USA).

## 4.  RESULTS

### 4.1.  TTV DNA detection and clinical and biochemical parameters of elderly subjects

Of 379 patients, 273 (72%) showed TTV DNA positivity in PMNLs. Table 1 outlines the baseline characteristics of the study population, grouped by TTV detection. TTV-positive subjects had a very slightly higher mean age than the TTV-negative group. No significant differences were observed for gender distribution, BMI, white blood cell count, erythrocytes number, haemoglobin, CRP, creatinine, albumin, fasting glucose and lipid serum levels. Conversely, the percentage of lymphocytes was increased, while neutrophils decreased in TTV positive subjects as compared to the negative ones (Table1, $p<0.01$). The prevalence of hypertension, cardiovascular diseases and type 2 diabetes was similar between the two classes of subjects, while an association between CMV serostatus and TTV DNA positivity was found and 76.4% of subjects were positive for both  viruses (Table1, $p<0.001$).

**4.2. TTV DNA load association with age, albumin, leukocytes, NK cell activity, inflammatory markers and CMV IgG antibody titer**

TTV levels were measured in PMNLs samples from the 273 subjects found positive for viral DNA presence. Overall, mean TTV load was 3.0 log copies per µg of total DNA extracted but, among individual subjects, it varied widely, ranging from 1.1 to 5.4 log DNA copies (confidence interval: 2.9 – 3.1 log TTV DNA copies per µg of total DNA extracted). In 65/273 subjects (24%), values >4 log DNA copies/µg of total DNA were measured, which represents a very high virus load, considering that the mean TTV levels measured in healthy blood donors range from 1 to 3 log TTV copies per ml of plasma or serum (Maggi et al., unpublished results).

TTV DNA loads were slightly, but significantly positively associated with age (R=0.267, p<0.001), independent of the sex of the subject (Fig. 1, females: R=0.283, p<0.001; males: R=0.264, p<0.001). Linear regression correcting for age evidenced a slight negative association among TTV DNA loads, albumin and NK cell activity (Fig. 2A: R = -0.244, p<0.0001; Fig. 2B: R = -0.269, p<0.05). A slight positive correlation was found among TTV DNA loads and monocytes in females (Fig. 3A: R = 0.206, p<0.01) and IL8 in males (Fig. 3B: R = 0.178, p<0.05). No significant association among TTV DNA loads and CMV IgG antibody titer or other inflammatory markers (CRP, Cu/Zn ratio and IL-6) (Fig. 2S, Fig. 3S, Fig. 4S, Fig. 5S, Supplementary material) or between TTV DNA load and other leukocytes populations (neutrophils and lymphocytes) was observed (Fig. 6S, Supplementary materials). Moreover, we observed a significant positive correlation between TTV DNA loads in PMNL and in plasma samples (log-transformed data) in a subgroup of 50 elderly subjects (N=50; 36 females and 14 males mean age: 80.4 ± 9.7) (R=0.571, P<0.0001). Of these fifty subjects, six (12%) had TTV negative plasma samples (Fig. 7S, Supplementary materials).

**4.3. Survival analysis**

Based on the Kaplan-Meier method, survival at three years differed significantly by TTV load (log-rank test: p < 0.0001; Fig. 4). The proportion of patients that died after three years of follow-up was estimated as 21.9% for patients with TTV DNA copies ≥ 4.0 log and 5.4% for patients with TTV copies < 4.0 log.

The Cox proportional hazards model revealed that, after controlling for age, sex, Cu/Zn, CRP, triglycerides, total cholesterol, albumin and glucose serum levels, the three year

mortality risk of elderly subjects increased significantly when TTV DNA copies were ≥ 4.0 log. (HR = 4.78; 95% CI: 1.70-13.44). Other factors independently associated with three year mortality were: age (HR = 1.07; 95% CI: 1.01-1.15), Cu/Zn (HR = 4.94; 95% CI: 1.62-15.07), CRP (HR = 0.69; 95% CI: 0.56-0.87) and serum albumin (HR = 0.09; 95% CI: 0.02-0.41) (Table  2). Interestingly, the Cox proportional hazards analysis showed a stronger association between TTV DNA copies ≥ 4.0 log and three year mortality risk when CMV IgG antibody titer was included in the model  (HR = 9.83; 95% CI: 2.48-38.97; N=343 subjects) (Table 1S , Supplementary material). Moreover, a slight association with three year mortality risk was also confirmed using the continuous variable TTV DNA levels (log-transformed data) in the Cox proportional hazards regression, correcting for age, sex, Cu/Zn, CRP, triglycerides, total cholesterol, albumin and  glucose serum levels (HR = 1.62; 95% CI: 1.05-2.51) (Table 2S , Supplementary material).

### 4.4.  TTV miRNA detection and quantification.

TTV miRNAs were measured in plasma exosomes, microvesicles released by the cells to transfer signalling competent proteins and functional microRNAs to other cells playing a role in cell-cell communication and in the modulation of immune response (Schorey et al., 2015) also during virus infection, as even suggested for miRNA-tth8  (Vignolini et al., 2016; Kincaid et al., 2013 ). Plasma exosome samples of 103 elderly subjects were examined for presence and loads of miRNA t1a, t3b, and tth8 belonging to TTV genogroups 1, 3, and 5, respectively. Overall, blood samples of 30 subjects (29%) contained at least one TTV miRNA, while only 3 samples revealed the simultaneous presence of two miRNAs (2%). miRNA-t3b was detected in blood of 28% of subjects at mean level of 566 copies per ng of RNA (± standard error (SE): 156 copies per ng of RNA), followed by miRNA-tth8 in 5% (mean level ± SE: 720 ± 279 copies per ng of RNA). No elderly subject was found to be positive for the presence of TTV miRNA-t1a (Table 3S Supplementary material).

### 4.5.   Automatic linear modelling for variables independently associated with TTV DNA loads and miRNA-t3b

According to the automatic linear modelling, we have categorized the top 5 important predictors of  miRNA-t3b variations in the plasma of elderly subjects as follow: triglycerides, monocytes, CRP, IL6 and lymphocyte percentage (Fig. 5A). To confirm the independent contributions of the variables to miRNA-t3b and TTV DNA load, a multiple linear regression model was performed (Table 4S Supplementary materials). The variability in the miRNA-t3b was best explained by triglycerides, monocytes, CRP, and IL6, while TTV DNA load

variability was dependent on age, triglycerides and albumin levels (Fig. 5B, automatic linear modelling), although linear regression did not confirm triglycerides serum concentrations as predictor for TTV viremia (Table 5S Supplementary materials).

## 5. Discussion

Susceptibility to infections increases significantly with age and persistent virus infection is responsible for immune perturbations leading to changes in the T cell pool that contributes to immunosenescence (Hassouneh et al., 2016; Di Benedetto et al., 2015).

Indeed, subclinical infections with viruses of the herpesviridae family, particularly CMV, are very common during ageing and have been associated with accelerated immunosenescence, telomere length shortening, elevated pro-inflammatory response, increased morbidity and mortality (Pawelec, 2014; Dowd et al., 2017; Westman et al, 2014; Simanek et al., 2011). Moreover, persistent CMV infection has been also linked to increased TTV DNA loads (Haloschan et al., 2014). TTV is an opportunistic pathogen that is often associated with co-infections, idiopathic pulmonary fibrosis, sepsis, cancer (Rosa et al., 2017; Walton et al., 2014; Szládek et al., 2005), as well as with COPD severity (Feyzioğlu et al., 2014). It has been suggested that TTV plasma DNA level reflects the balance between a high daily virion generation rate and a host's clearance rate of over 90 % per day (Maggi et al. 2001) and, thus, indirectly reflects the antiviral immune response against TTV.

According to previous evidence, we observed a positive correlation between age and TTV viremia, suggesting a relationship between degree of immunosenescence and virus replication (Haloschan et al., 2014). These findings indicate a decreased antiviral immune response against chronic TTV infection with advancing age, supporting previous studies, showing an impaired immune response in the elderly (Pawelec et al.,2014). In fact, TTV genome replication seems to be controlled by the immune system and higher viral titers were found in individuals with immunodeficiencies, such as AIDS (Shibayama et al., 2001) or immunesuppression condition (Focosi et al., 2015). Although the significance of TTV load on increased risk of morbidity and mortality is still unclear, in the present study we show, for the first time, that TTV load in PMNLs is independently associated with all-cause mortality, after controlling for the main confounders related to death (Malavolta et al., 2010; Barger et al., 2016) including CMV IgG antibody titer (Table 2S, Supplementary Material) that was not associated with the increased risk mortality in contrast to a previous study (Simaneket al., 2011). The reason for this lack of association may be attributed to intrinsic

or environmental features of the Italian population involved in this study. Interestingly, most Italian centenarians where previously found to be positive for CMV (94.7%) and their offspring seem to be less susceptible to CMV-driven changes to the immune system (Sansoni et al. 2014).

Serum albumin levels represent a strong predictor of mortality in the elderly (Liu et al., 2015), as well as during some viral infections (Saroch et al., 2017; Lang et al., 2013) and this is consistent with the negative association among TTV viremia  and serum albumin concentrations observed in our cohort. Another result, supporting the implication of TTV load in the increased risk of mortality in our elderly population, may be represented by the positive correlation between TTV DNA and IL8 (although only in females), because higher IL-8 plasma levels have been associated with an increased risk of long-term all-cause mortality in patients with coronary-heart-disease (Cavusoglu et al., 2015). However, no significant association between TTV load and markers of systemic inflammation (i. e. Cu/Zn ratio , IL6, CRP; Supplemetary Materials), previously demonstrated as strong predictors of mortality (Malavolta et al., 2010; Puzianowska-Kuźnicka et al., 2016), was observed in our cohort.

Moreover, a possible exhaustion of the CD4/CD8 clonal repertoire, during persistent TTV infection, could explain the decreased survival in elderly population as observed in octo-nonagenarian subjects with CMV chronic infection (Hadrup et al., 2006). In fact, similarly to CMV infection, it is likely that  a constant immune surveillance, necessary to keep the TTV under control, might lead to clonal expansions of TTV-specific T cells, at the expense of a naïve/memory highly diversified repertoire (Di Benedetto et al., 2015). In our study, we have no data on TTV-specific T cells subsets, but we found a negative correlation between TTV reactivation and NK cell activity. NK cells exert a critical role in the control of viral infections and the age-related NK cell impairment predisposes to virus reactivation and persistence (Brandstadter and Yang 2011), therefore it is not surprising that elderly individuals with impaired NK cell activity presented higher TTV viral loads. Furthermore, TTV encodes for miRNA targeting N-myc interactor (NMI) (Kincaid et al., 2013), a transcription cofactor regulating the activity of STAT members in response to interferon- γ (IFN-γ) and cytokine signaling (Zhu et al., 1999). Consequently, TTV miRNA could mediate immune evasion leading to a reduced IFN-γ production by NK cells with less efficient DC maturation and T cell polarization (Hazeldine and Lord 2013). Interestingly, a recent investigation shows a similar mechanism of viral immune escape from IFN-γ responses in CMV infection (Feng et al., 2018).

Considering that persistent antigen stimulation leads to T-cell exhaustion and seems to favor telomere attrition during chronic viral infections (Bellon et al., 2017; Dowd et al., 2017), and because a close correlation has been demonstrated between granulocyte and lymphocyte telomere length (TL) (Kimura et al., 2010), we evaluated the association between TL in PBMCs and TTV viral load to demonstrate the role of TTV chronic infection in immunosenescence. However, we did not find an association between TL in PBMCs and TTV loads in a subgroup of 87 subjects (Fig 1S Supplementary materials), that might also depend on the small sample size.

We have also investigated TTV miRNA that might be involved in viral immune evasion. Conversely to previous evidence (Vignolini et al., 2016), we did not find any blood sample positive for miRNA-t1a of TTV group 1, besides miRNA-t3b and miRNA–tth8 showed a frequency in elderly people lower than the one found in healthy donors previously examined (Vignolini et al. 2016; Table 5S, Supplementary materials). To date, role and significance of TTV miRNAs expression is yet essentially unknown, but our results demonstrate that significant differences could appear in production of some TTV miRNAs when different groups of subjects are compared to each other. What these findings mean is still unexplored, but the different expression of these miRNAs in relation to the immune status of differently aged subjects encourages further studies in this direction.

In addition, no correlation between TTV DNA loads and miRNA-t3b was found. This finding might be related to the different samples used (PMNLs for TTV DNA load and plasma for miRNA-t3b), but also depend from the circumstance that miRNA-t3b may be mainly produced by TTV localized in body districts different from PMNLs.

We have also demonstrated by an automatic linear modelling, that the levels of miRNA-t3b are inversely related to triglycerides, monocytes and CRP, as well as positively correlated to IL6.

Therefore, lipid levels, CRP and monocytes could play a role in suppressing virus infection and miRNA production, as observed during HCV or HIV infection (Atta et al., 2012; Dai et al., 2008; Kuwata et al., 2007), whereas IL6 might be upregulated by viral miRNA as observed in other viral diseases (Qi et al., 2013). However, the regulatory role of TTV miRNAs in inflammatory pathways is unknown and deserves specific functional investigations.

This study presents a limitation consisting in  the small sample size, therefore the association between TTV DNA load and mortality needs to be confirmed in larger cohorts of elderly individuals.

In conclusion, this study supports a role of TTV in immunesenescence and increased risk for all-cause mortality in a cohort of Italian elderly people. TTV may represent an additional virus that establishes latency after primary infection and reactivates in aging when the immune system is compromised. Further research examining if TTV may be causally related to immunosenescence and age-related diseases is warranted.

**Table 1 Laboratory parameters in elderly subjects**

| | TTV negative (n.106) | TTV positive (n.273) | P value |
|---|---|---|---|
| Age (yrs) | 75.0±6.7 | 77.5±9.0 | 0.012 |
| Females (%) | 58.5% | 57.1% | NS |
| BMI | 25.1 | 24.6 | NS |
| WBC ($10^3$/µl) | 7.1±4.9 | 6.7±3.8 | NS |
| Lymphocytes (%) | 27.7±7.8 | 30.1±8.6 | 0.007 |
| Neutrophils (%) | 61.1±8.4 | 58.6±9.4 | 0.009 |
| Erythrocytes ($10^6$/ µl) | 4.6±0.5 | 4.5±0.5 | NS |
| Hemoglobin (g/dl) | 13.8±1.5 | 13.5±1.6 | NS |
| CRP (pg/ml) | 0.74 ±1.85 | 0.77±1.94 | NS |
| Creatinine (mg/dl) | 0.94±0.42 | 1.00±0.27 | NS |
| Albumin (g/dl) | 3.75±0.43 | 3.62±0.38 | NS |
| Glycemia  (mg/dl) | 103±29 | 100±29 | NS |

| | | | |
|---|---|---|---|
| TG (mg/dl) | 116±48 | 122±77 | NS |
| TC (mg/dl) | 201±36 | 205±43 | NS |
| HDL-C (mg/dl) | 55±14 | 53±17 | NS |
| Hypertension | 59.1% | 61.7% | NS |
| CVD diseases | 34.4% | 36.2% | NS |
| Diabetes mellitus | 17.6 | 13.2 | NS |
| CMV seropositivity* | 23.6% | 76.4% | 0.001 |

Data are mean ± standard deviation

The laboratory parameter analysis were adjusted for age and gender

WBC, white blood cells; CRP, C-reactive protein; TG, triglycerides; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; CVD cardiovascular diseases

* analyzed in 343 subjects

**Table 2. Multivariate cox regression analysis of TTV DNA loads in PMNLs and all-cause mortality in elderly subjects**

|  | HR | CI 95% | P value |
|---|---|---|---|
| **TTV ≥ 4.0 DNA log copies/??g** | 4.78 | 1.70-13.44 | 0.003 |
| **Age** | 1.07 | 1.01-1.15 | 0.026 |
| **gender** | 0.86 | 0.28-2.64 | 0.796 |
| **Cu/Zn** | 4.94 | 1.62-15.07 | 0.005 |
| **CRP** | 0.69 | 0.56-0.87 | 0.001 |
| **TG** | 1.00 | 0.99-1.01 | 0.540 |
| **TC** | 0.99 | 0.98-1.01 | 0.714 |
| **Albumin** | 0.09 | 0.02-0.41 | 0.002 |
| **Glycemia** | 0.98 | 0.95-1.02 | 0.350 |

Cox regression model for all-cause mortality (N = 379) adjusted for age, gender, Cu/Zn (Copper Zinc ratio), CRP (C-reactive protein), TG, triglycerides, TC, total cholesterol, albumin and fasting glucose levels

**Fig 1 Correlation between TTV DNA loads in PMNL and age in elderly subjects**

Pearson Correlation between TTV DNA loads (log-transformed data) and age was analyzed both in males and females). Viral loads were expressed as the number of viral genomic copies per μg of genomic DNA extracted by PMNL (Copies/ μg). R coefficient and P value are shown.

**Fig 2 Linear regression between TTV DNA loads in PMNL serum albumin levels and NK cell activity in elderly subjects**

A negative linear regression between TTV DNA loads (log-transformed data) and serum albumin levels (Ln-transformed data) after adjusting for age (Fig. 2A). A negative linear regression between TTV DNA loads (log-transformed data) and NK cell activity (Ln-transformed data) after adjusting for age in a subgroup of 71 elderly subjects (27 males: mean age 76.8 ± 7.9; 44 felales, mean age: 76.1 ± 8.4) (Fig. 2B) . Viral loads were expressed as the number of viral genomic copies per μg of genomic DNA extracted by PMNLs (copies/ μg). Standardized Beta coefficient and P value are shown.

**Fig 3 Linear regression between TTV DNA loads in PMNL , monocytes and IL8 in elderly subjects**

Significant positive linear regression between TTV DNA loads (log-transformed data) and monocyte percentage (Ln-transformed data) after adjusting for age in females (Fig. 3A). Significant positive linear regression between TTV DNA loads (log-transformed data) and IL8 (Ln-transformed data) after adjusting for age in males (Fig. 3B).

Viral loads were expressed as the number of viral genomic copies per million of cells (copies/M). Standardized Beta coefficient and P value are shown.

**Fig. 4 Kaplan-Meier survival estimates according to TTV DNA loads in elderly patients**

TTV cat 1 = TTV log copies/µg <4.0; TTV cat 2= TTV log copies/ µg ≥ 4.0. This figure shows Kaplan-Meier plots comparing all-cause-mortality between TTV log copies/µg <4.0 (blue line) and TTV log copies/µg ≥4.0 (green line)

**Fig. 5 Coefficients (SPSS automatic Linear modeling) for miRNA-t3b and TTV DNA loads**. These charts display the intercept first and then sorts effects from top to bottom in decreasing predictor importance. Within effect containing factors, coefficients are sorted by ascending order of data values. The width of connecting lines in the diagram reflects the coefficient significance, with greater line width corresponding to more significant coefficients (smaller p values).

**Conflict of Interest**: On behalf of all authors, the corresponding author states that there is no conflict of interest.

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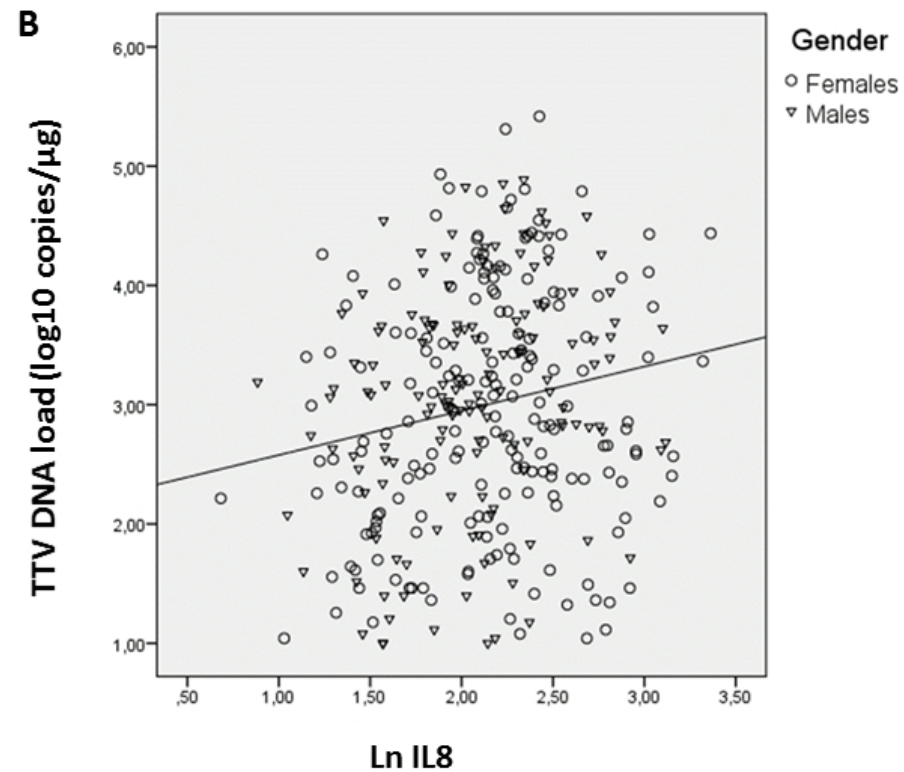
Females R=0.283; p<0.001
Males R=0.264 p<0.001
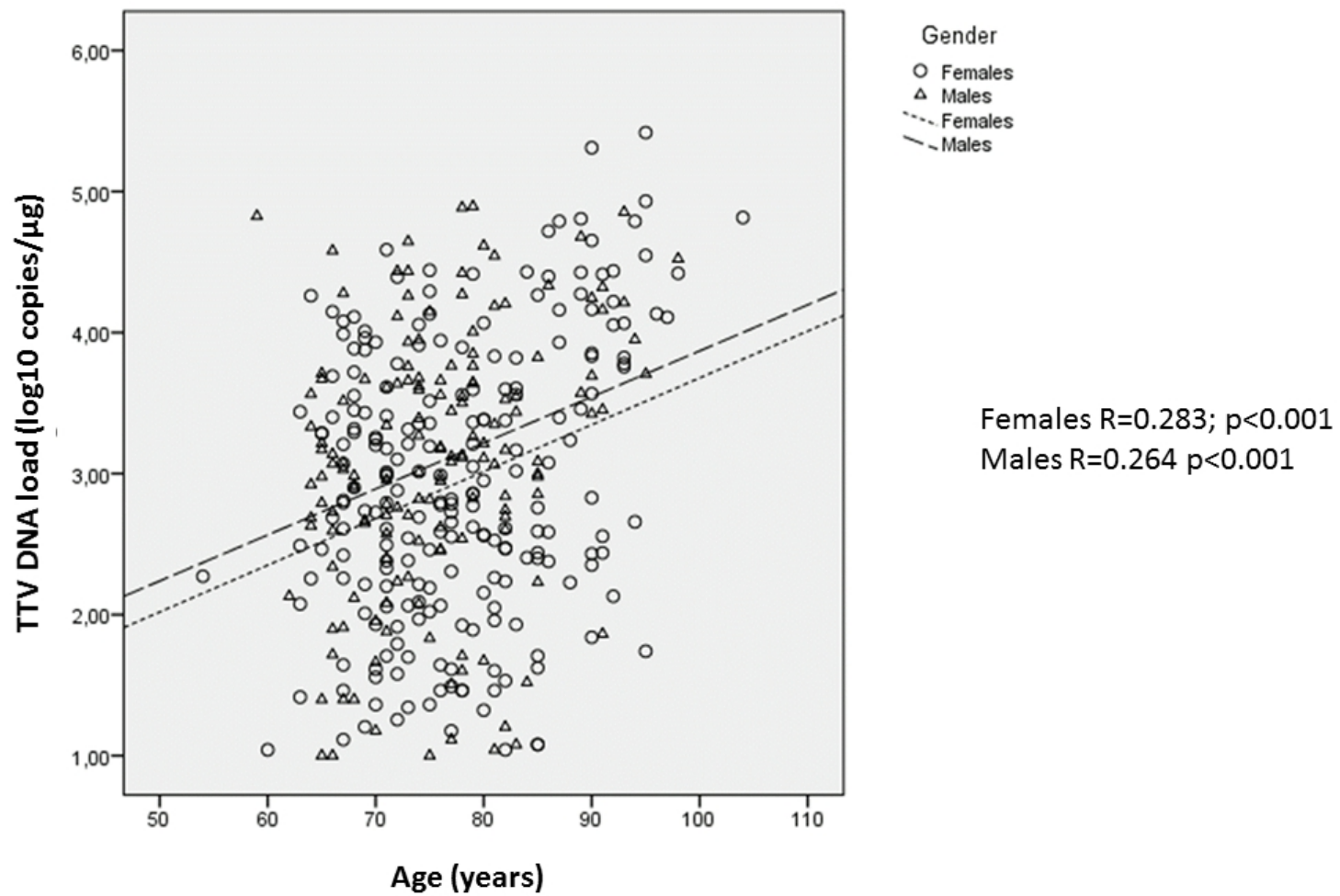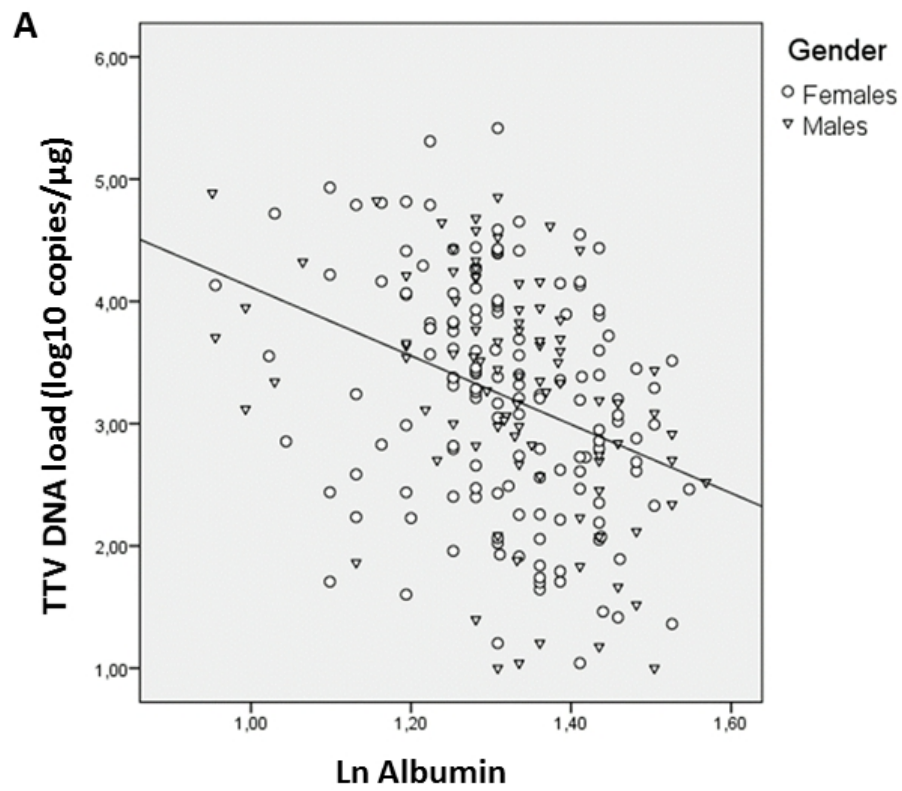
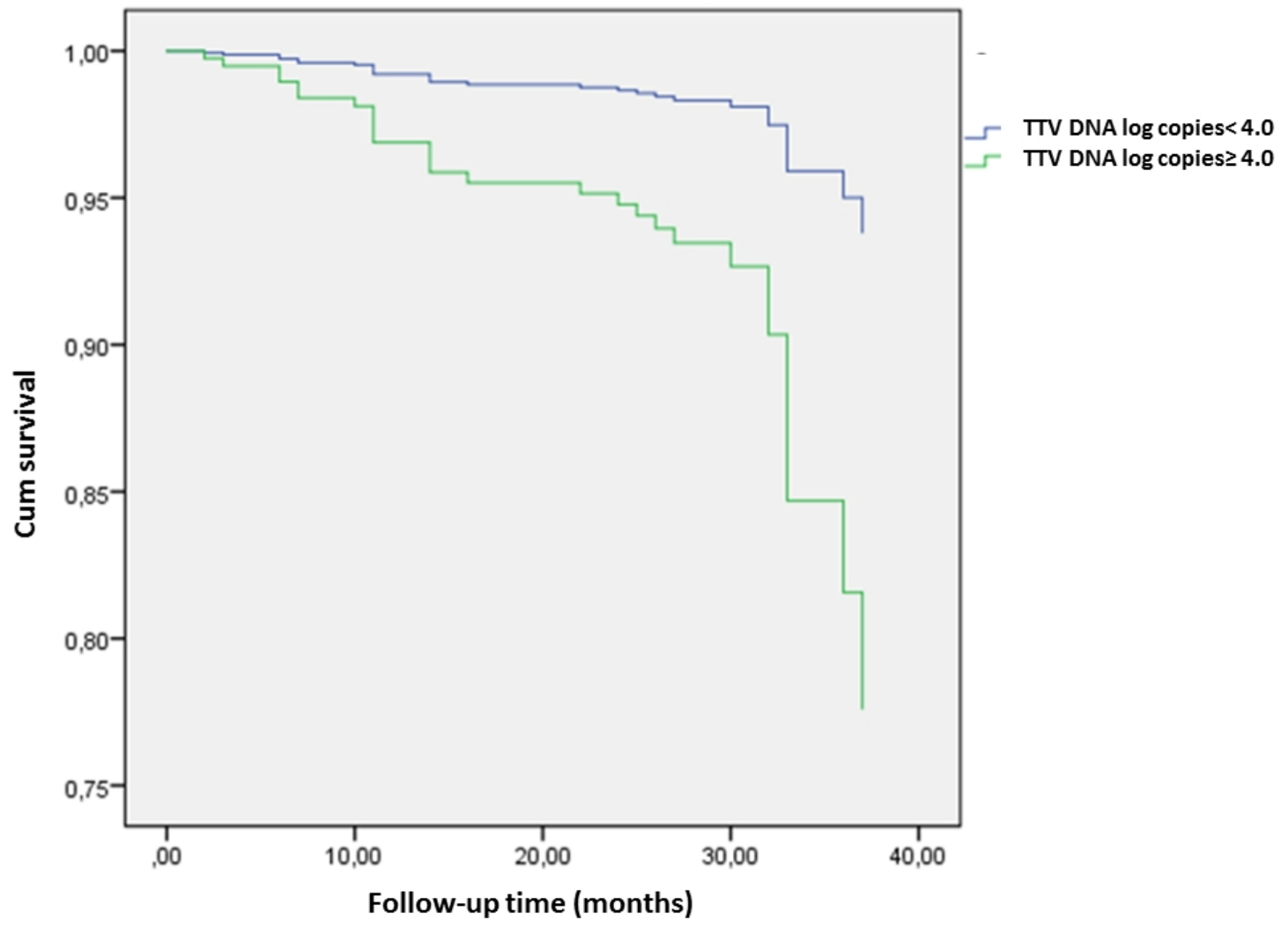Beta coefficient= -0.244   p<0.0001
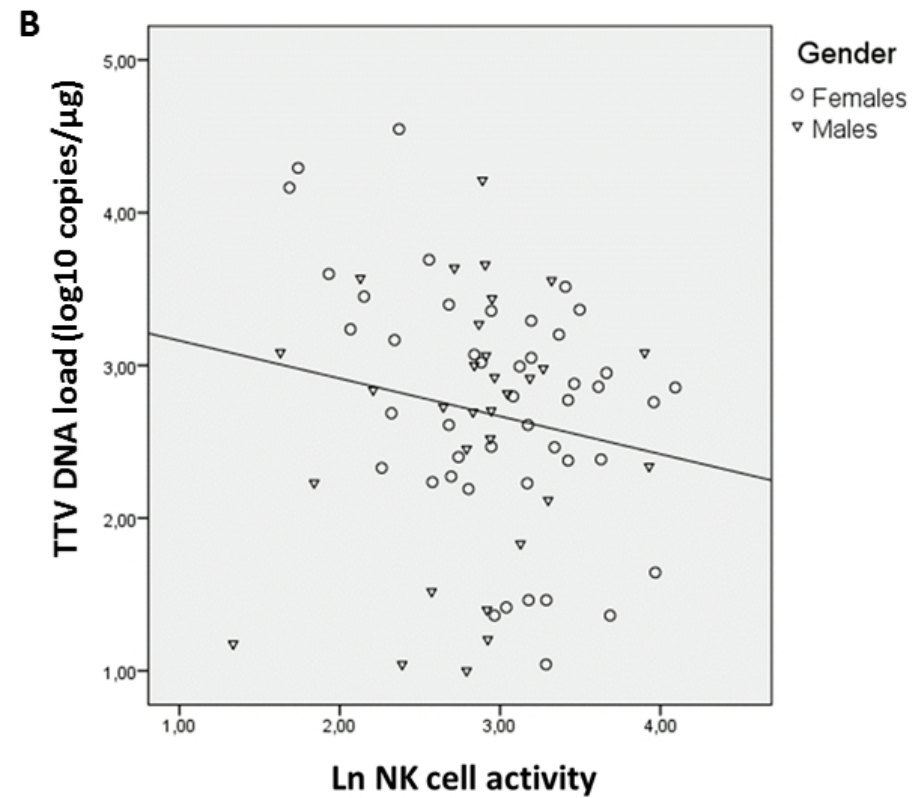
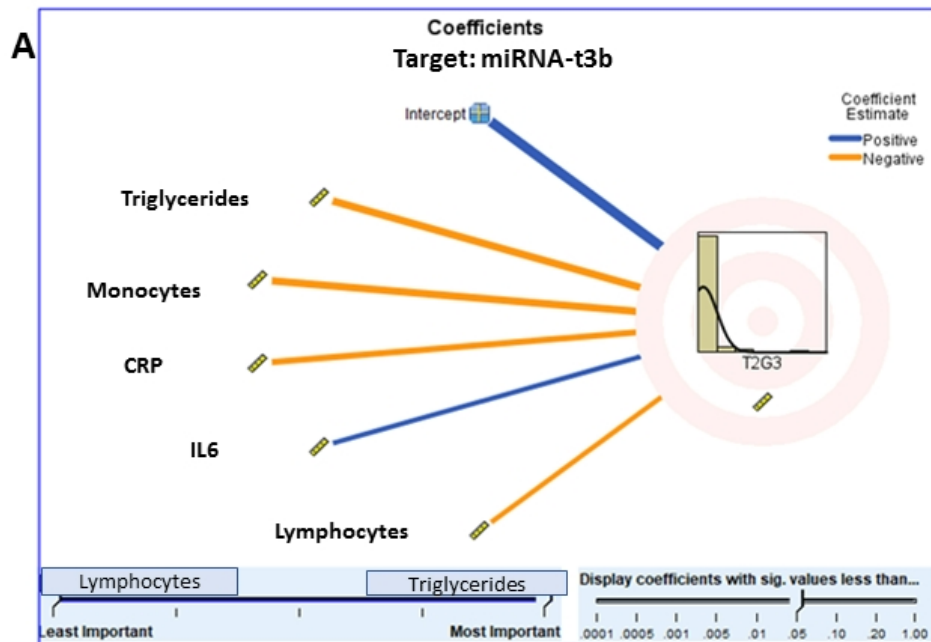Beta coefficient= -0.269   p<0.05

**A**
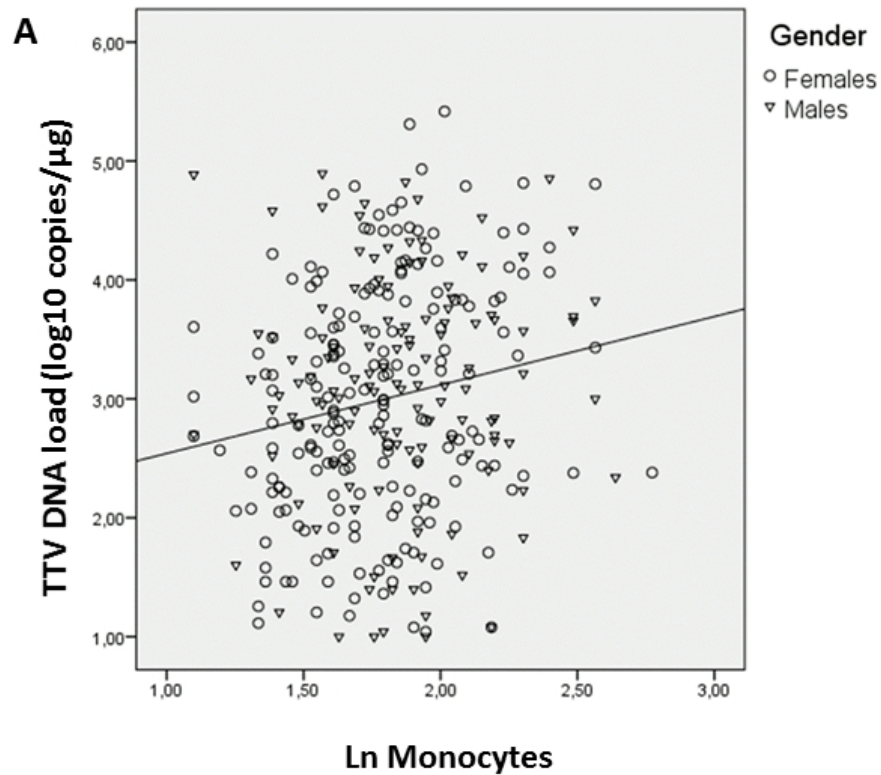
Males: Beta coefficient= 0.014 p=0.88
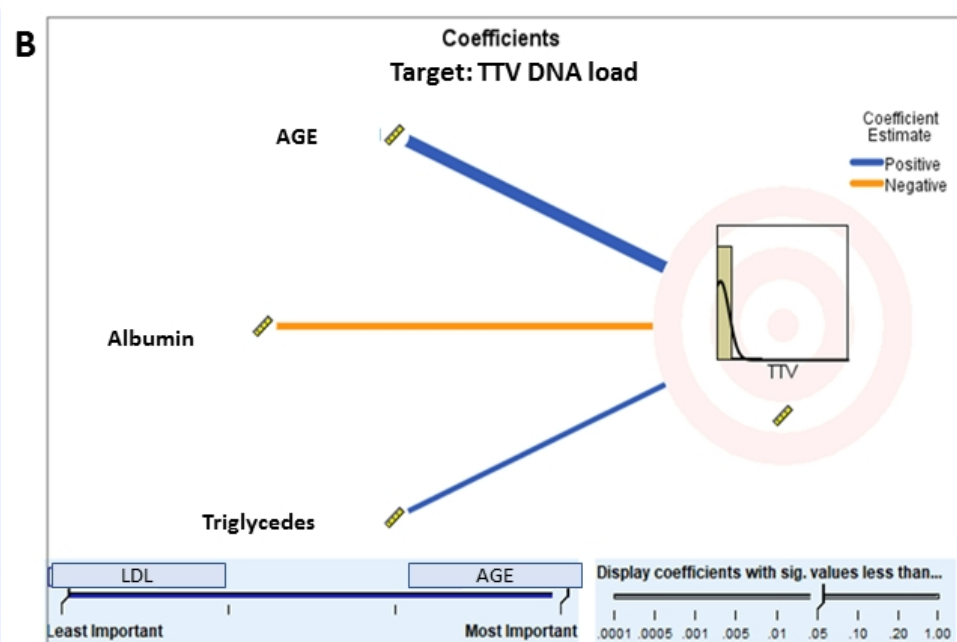Females: Beta coefficient= 0.206 p=0.009

**B**

Males: Beta coefficient= 0.178 p=0.014
Females: Beta coefficient= 0.168 p=0.08

**A**

Coefficients
Target: miRNA-t3b

| Model Term | Coefficient ▶ | Sig. | Importance |
|---|---|---|---|
| Intercept | 1,547.914 | .000 | |
| Triglycerides | -5.172 | .002 | 0.276 |
| Monocytes | -90.758 | .004 | 0.241 |
| CRP | -287.906 | .006 | 0.214 |
| IL6 | 17.435 | .023 | 0.147 |
| Lymphocytes | -14.226 | .037 | 0.122 |

**B**

Coefficients
Target: TTV DNA load

| Model Term | Coefficient ▶ | Sig. | Importance |
|---|---|---|---|
| AGE | 589.612 | .000 | 0.642 |
| Albumin | -7,092.953 | .009 | 0.160 |
| Triglycerides | 46.556 | .015 | 0.142 |

# Torquetenovirus (TTV) load predicts mortality in Italian elderly subjects

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**Table 1S Multivariate cox regression analysis of TTV DNA loads in PMNLs and all-cause mortality after adjustment for CMV IgG antibody titer in elderly subjects**

| | HR | CI 95% | P value |
|---|---|---|---|
| **TTV ≥ 4.0 DNA log copies/??g** | 9.83 | 2.48-38.97 | 0.001 |
| **Age** | 1.06 | 0.98-1.14 | 0.125 |
| **gender** | | | |
| **CMV IgG antibody titer (log values)** | 187.11 | 0.63-55215 | 0.071 |
| **Cu/Zn** | 99.44 | 5.12-1928 | 0.002 |
| **CRP** | 0.70 | 0.44-1.13 | 0.151 |
| **TG** | 1.62 | 0.38-6.92 | 0.512 |
| **TC** | 0.21 | 0.007-6.67 | 0.380 |
| **Albumin** | 0.012 | 0.001-1.46 | 0.071 |
| **Glycemia** | 0.099 | 0.001-6.63 | 0.281 |

Cox regression model for all-cause mortality (N = 343) adjusted for age, gender, CMV IgG antibody titer, Cu/Zn (Copper Zinc ratio), CRP (C-reactive protein), TG, triglycerides, TC, total cholesterol, albumin and fasting glucose levels

**Table 2S Multivariate cox regression analysis of TTV DNA loads (continuous variable) in PMNLs and all-cause mortality in elderly subjects**

| | HR | CI 95% | P value |
|---|---|---|---|
| **TTV DNA log copies/??g** | 1.62 | 1.05-2.51 | 0.030 |
| **Age** | 1.10 | 1.02-1.18 | 0.006 |
| **gender** | 1.01 | 0.30-3.37 | 0.98 |
| **Cu/Zn** | 23.52 | 1.94-284.9 | 0.013 |
| **CRP** | 0.88 | 0.59-1.28 | 0.504 |
| **TG** | 1.84 | 0.50-6.76 | 0.359 |
| **TC** | 0.506 | 0.028-9.127 | 0.64 |
| **Albumin** | 0.003 | 0.00-0.285 | 0.012 |
| **Glycemia** | 0.061 | 0.002-1.998 | 0.116 |

Cox regression model for all-cause mortality (N = 379) TTV DNA copies was slightly but significantly associated with three year mortality after adjustement for age, gender, Cu/Zn

**Table 3S** **TTV miRNAs expression in elderly participants**

| | Elderly subjects (n.103) | |
|---|---|---|
| | **N. of positive patients (%)** | **Mean load** |
| miRNa-t1a (TTV group 1) | 0% | - |
| miRNa-t3b (TTV group 3) | 28.2% | 565±841 |
| miRNa-tth8 (TTV group 5) | 4.8% | 720±624 |

A subgroup of subjects representative of the whole population were selected for the assay of  TTV miRNA expression in plasma

TTV miRNA were expressed as copies per ng of total RNA extracted ± standard deviation.

**Table 4S** **Multiple linear regression model among miRNA-t3b of TTV group 3 and variables independently associated after Automatic linear modelling**

Coefficients[a]

| Model | | Unstandardized Coefficients | | Standardized Coefficients | t | Sig. |
|---|---|---|---|---|---|---|
| | | B | Std. Error | Beta | | |
| 1 | (Constant) | 6839,024 | 1652,913 | | 4,138 | ,000 |
| | Lntryglycerides | -972,889 | 248,722 | -,487 | -3,912 | ,000 |
| | LnCRP | -317,369 | 78,388 | -,577 | -4,049 | ,000 |
| | LnIL6 | 650,575 | 240,146 | ,326 | 2,709 | ,010 |
| | LnMonocytes | -1258,752 | 283,372 | -,557 | -4,442 | ,000 |
| | LnLymphocytes | -613,271 | 280,223 | -,293 | -2,189 | ,034 |

**Table 5S** **Multiple linear regression model among TTV DNA loads in PMNL and variables independently associated after Automatic linear modelling**

Coefficients[a]

| Model | | Unstandardized Coefficients | | Standardized Coefficients | t | Sig. |
|---|---|---|---|---|---|---|
| | | B | Std. Error | Beta | | |
| 1 | (Constant) | 3,786 | 1,147 | | 3,300 | ,001 |
| | Age in years | ,029 | ,007 | ,283 | 4,416 | ,000 |
| | Lntriglycerides | -,071 | ,122 | -,035 | -,581 | ,561 |
| | LnAlbumin | -1,905 | ,519 | -,235 | -3,669 | ,000 |

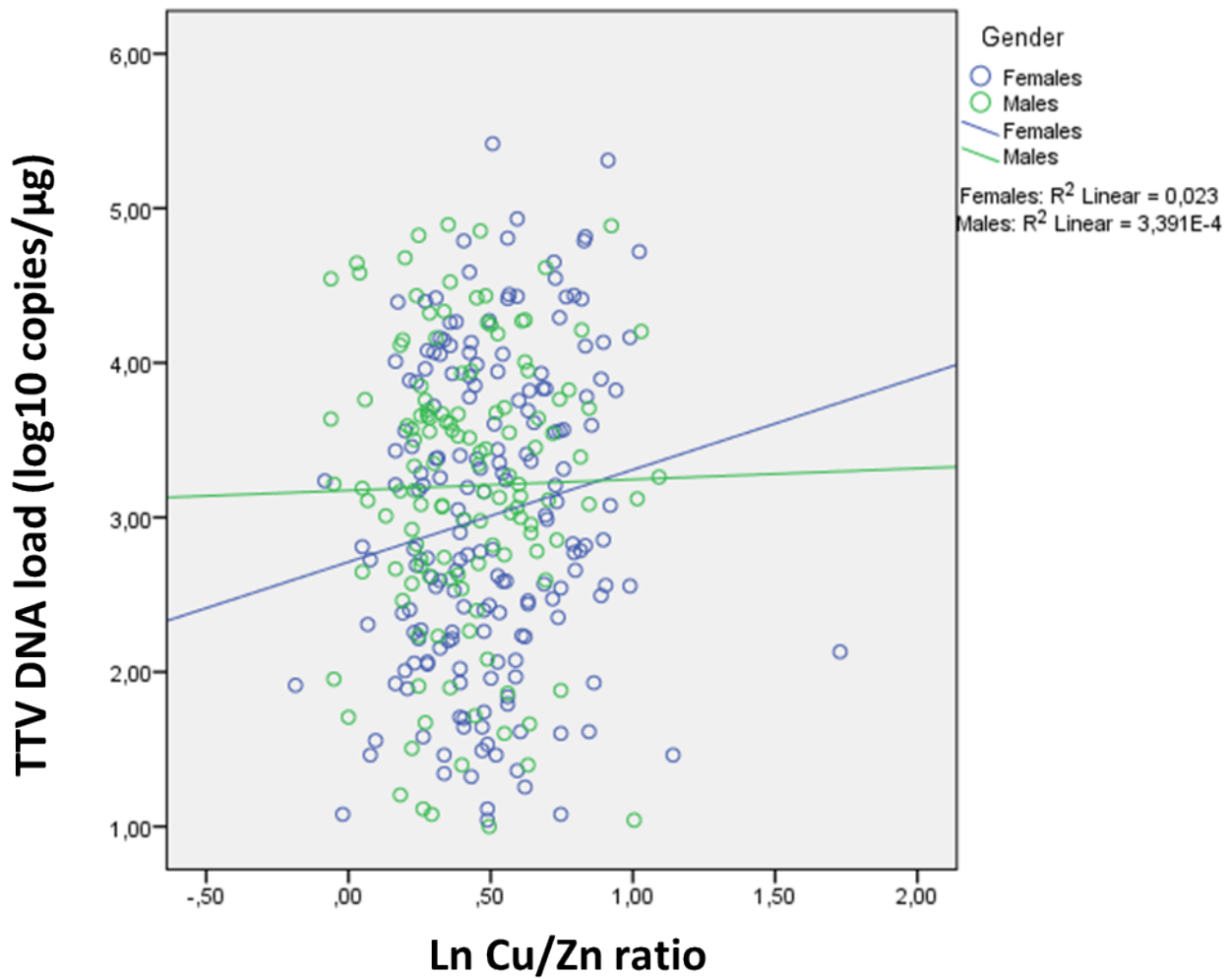a. Dependent Variable: log10TTV
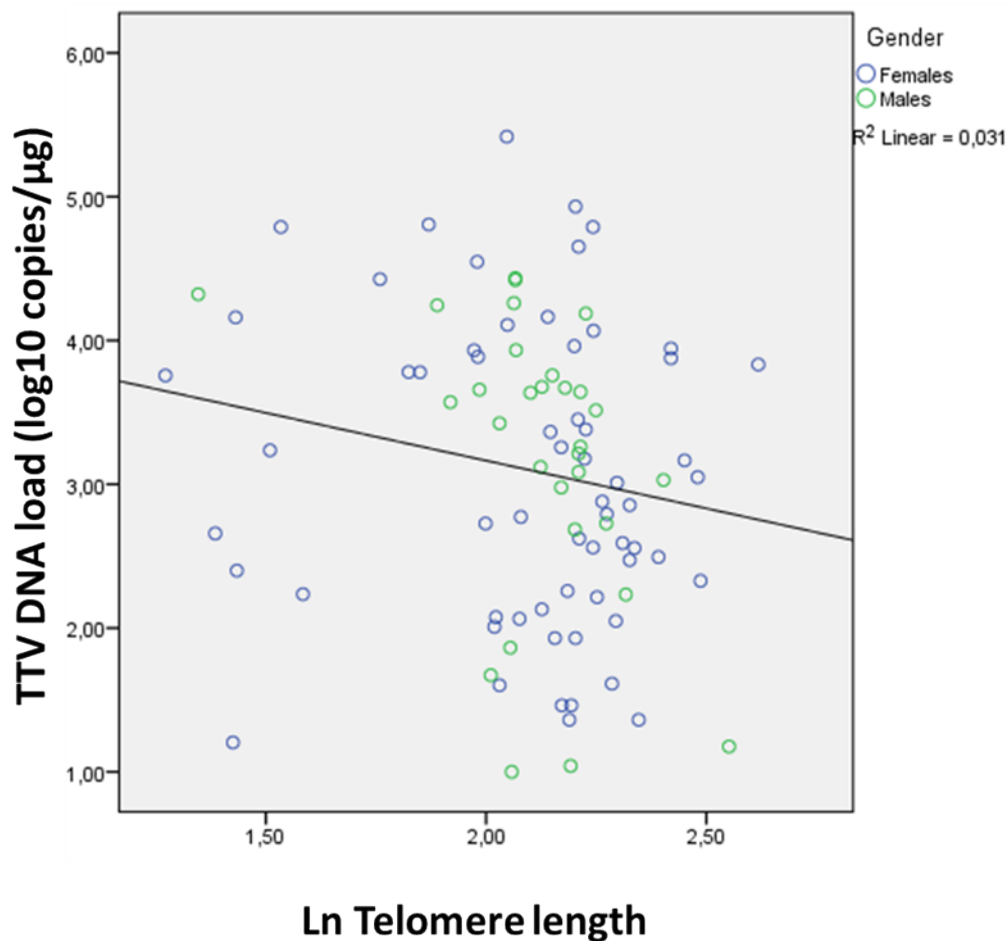
**Fig 1S** **Pearson correlation between TTV DNA loads in PMNL and telomere length in elderly subjects**

A negative correlation between TTV DNA loads (log-transformed data) and telomere length (Ln-transformed data) was observed (R=-0,219 P<0.05) in a subgroup of 87 elderly subjects. However, Linear regression after adjusting for age showed no significant association ( Beta coefficient:-0.-093 p=0.41). Telomere length was determined by flow-FISH methodology by using the Telomere PNA kit/FITC from Dako (Glostrup, Denmark) according manufacture's instruction. Viral loads were expressed as number of viral genomic copies per μg of genomic DNA extracted by PMNL (Copies/ μg).
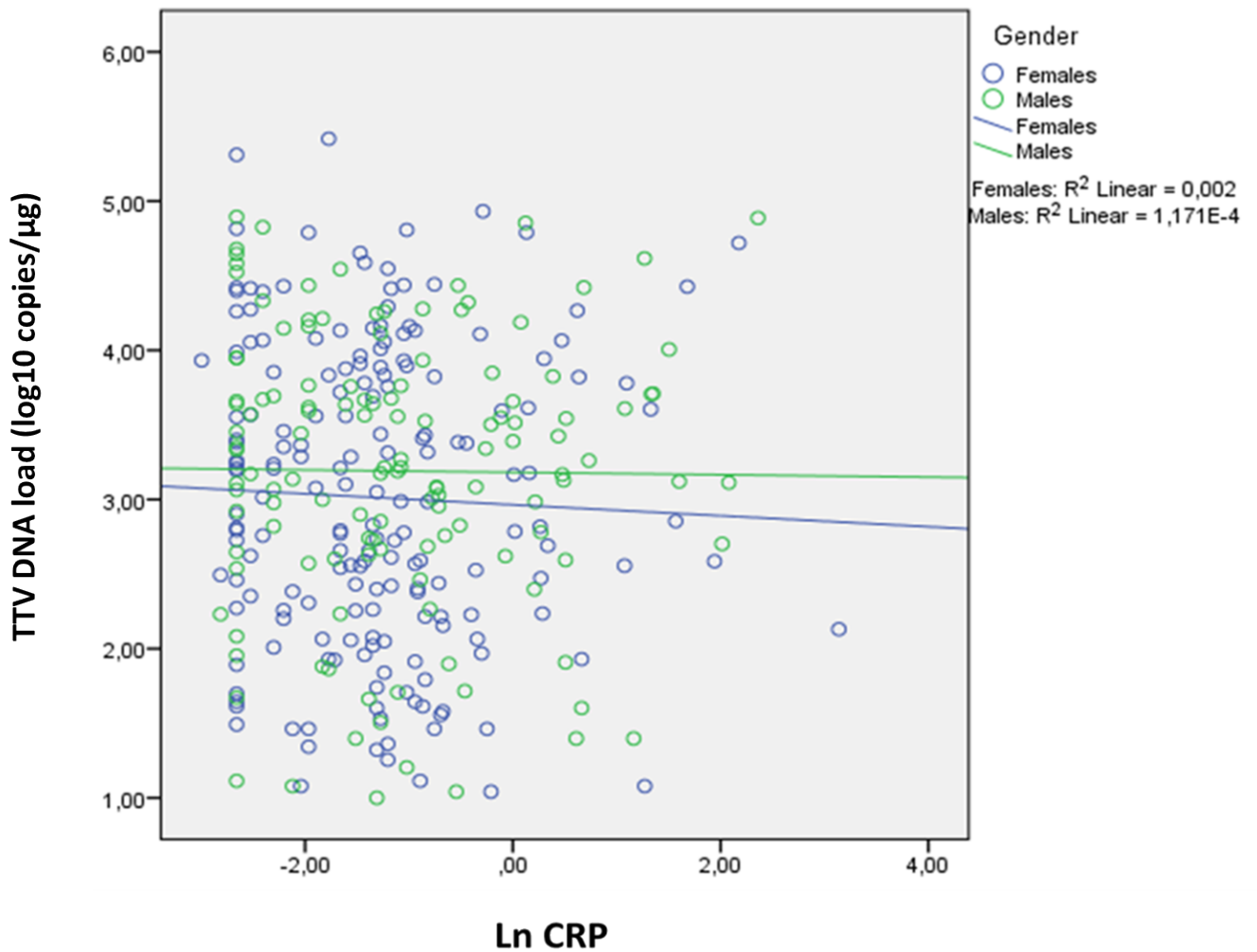
4
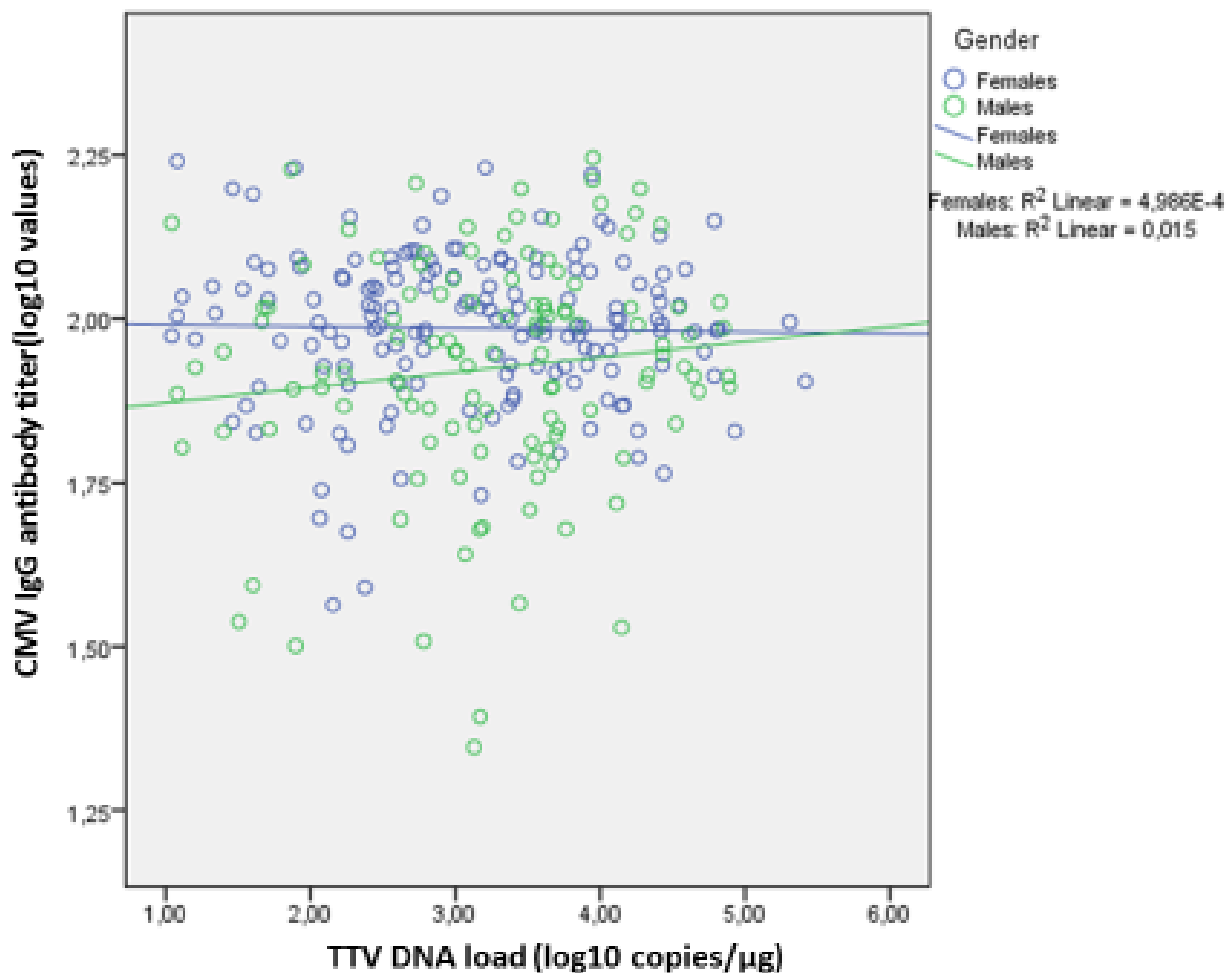
**Fig 2S Pearson correlation between TTV DNA loads in PMNL and CMV IgG antibody titer in elderly subjects**

No significant correlation between TTV DNA loads (log-transformed data) and CMV IgG antibody titer (Log-transformed data) [Total population (N=343) R=0.034, P=0.565; Females R=-0.022 p=0.77; Males R=0.122 P=0.189]. Linear regression after adjusting for age confirmed that there was no significant association (Beta coefficient:0.005 p=0.93). Viral loads were expressed as number of viral genomic copies per µg of genomic DNA extracted by PMNL (Copies/ µg).
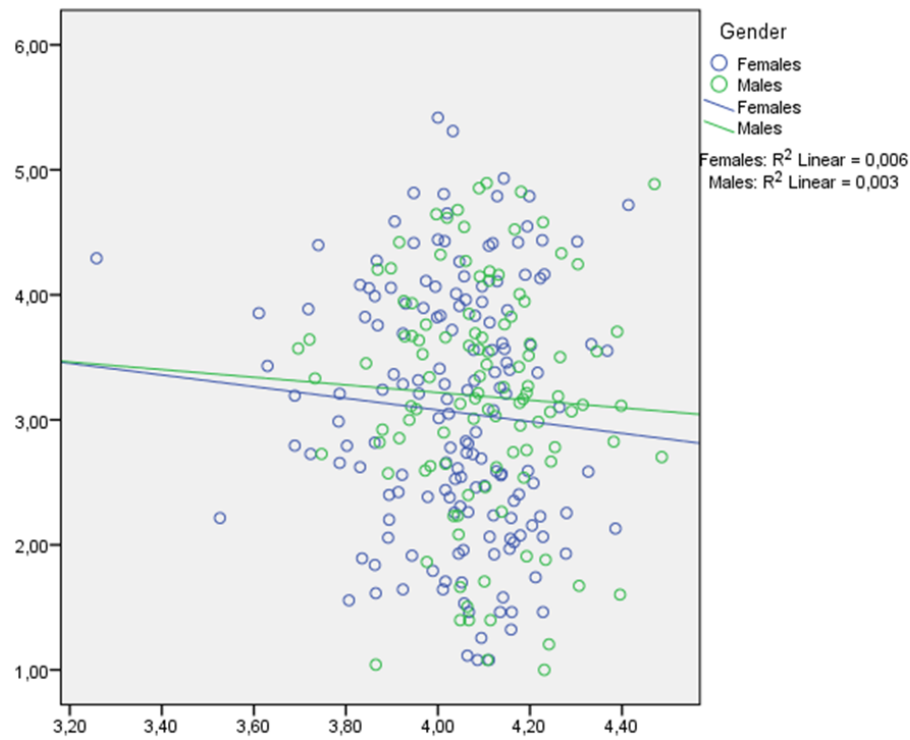
**Fig 3S Pearson correlation between TTV DNA loads in PMNL, and Cu/Zn ratio in elderly subjects**

Significant positive correlation between TTV DNA loads (log-transformed data) and Cu/Zn ratio (Ln-transformed data) (Total population R=0.115, P<0.05; Females R=0.169 p=0.019; Males R=0.007 P=0.937). However, Linear regression after adjusting for age showed no significant association (Beta coefficient:0.029 p=0.53). Viral loads were expressed as number of viral genomic copies per µg of genomic DNA extracted by PMNL (Copies/ µg).
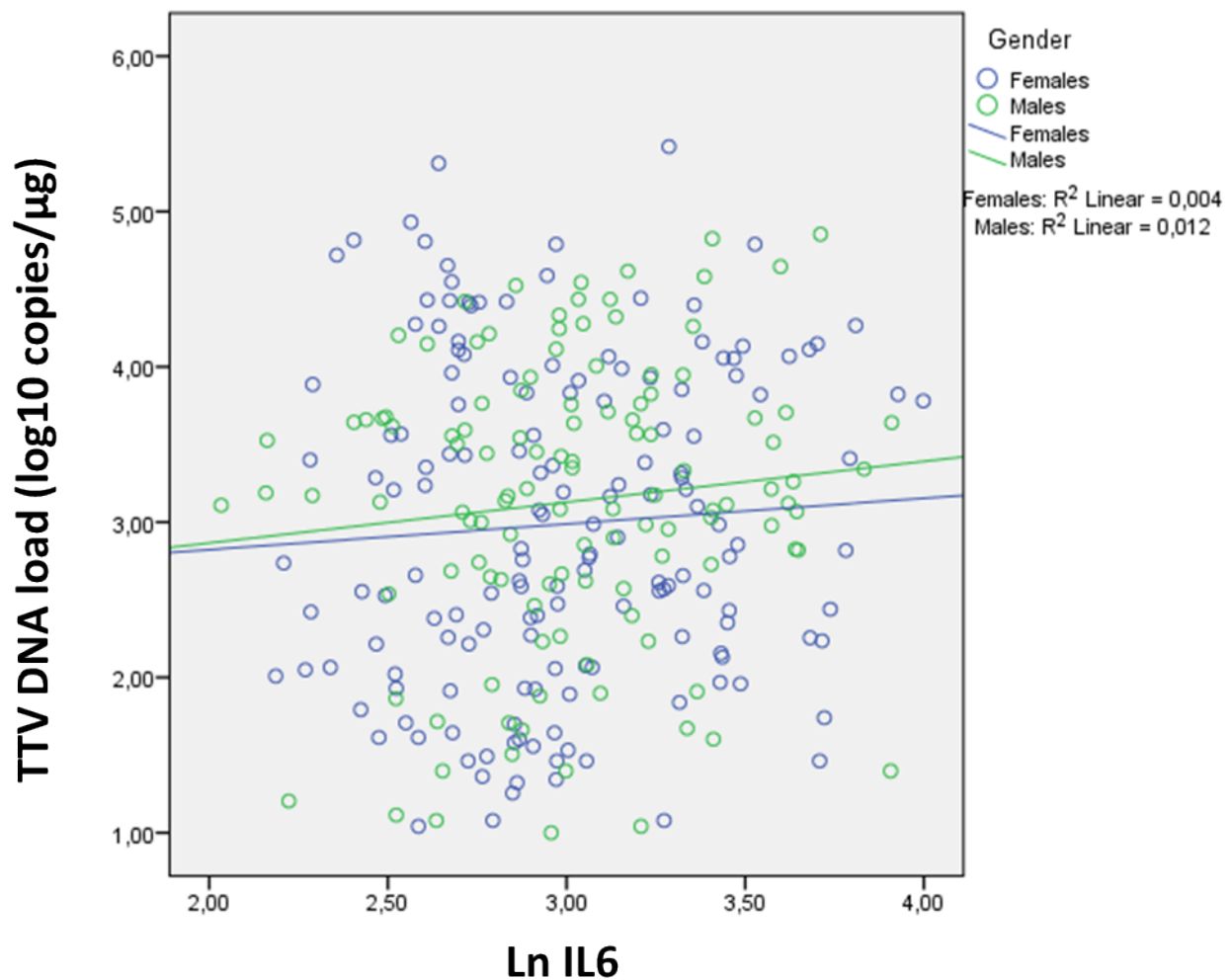
**Fig 4S** **Pearson correlation between TTV DNA loads in PMNL, and IL6 in elderly subjects**

Significant positive correlation between TTV DNA loads (log-transformed data) and IL6 (Ln-transformed data) (R=0.114, P<0.05). However, Linear regression after adjusting for age showed no significant association (Beta coefficient:0.093 p=0.078) Viral loads were expressed as number of viral genomic copies per µg of genomic DNA extracted by PMNL (Copies/ µg).
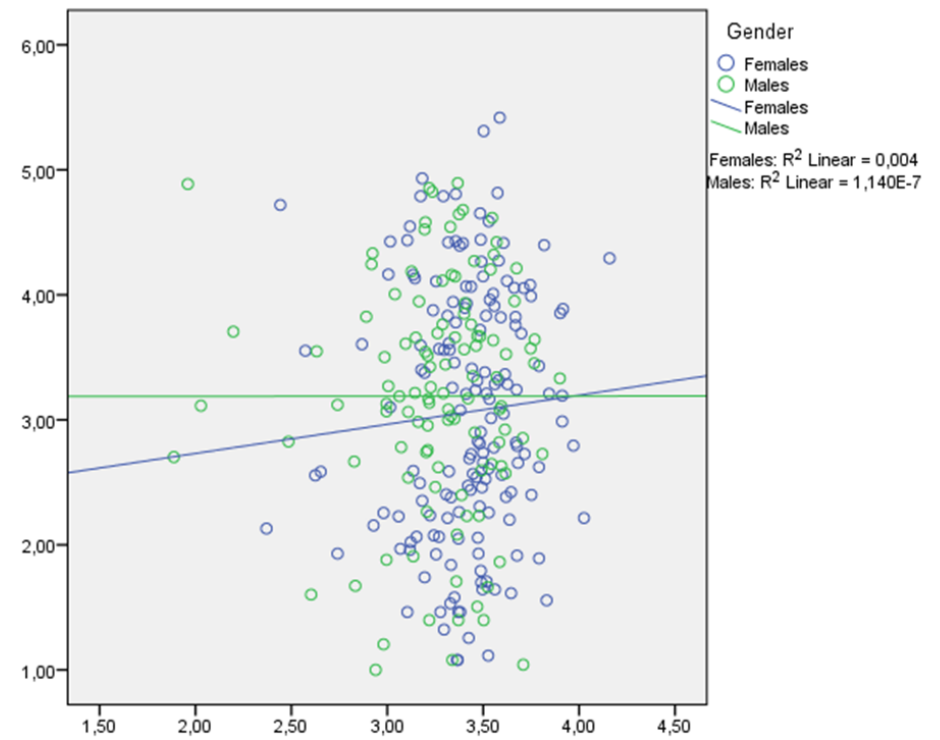
**Fig 5S** Pearson correlation between TTV DNA loads in PMNL , and CRP in elderly subjects

No significant correlation between TTV DNA loads (log-transformed data) and CRP (Ln-transformed data) (R=-0.19, P=0.735) was observed. Viral loads were expressed as number of viral genomic copies per µg of genomic DNA extracted by PMNL (Copies/ µg)
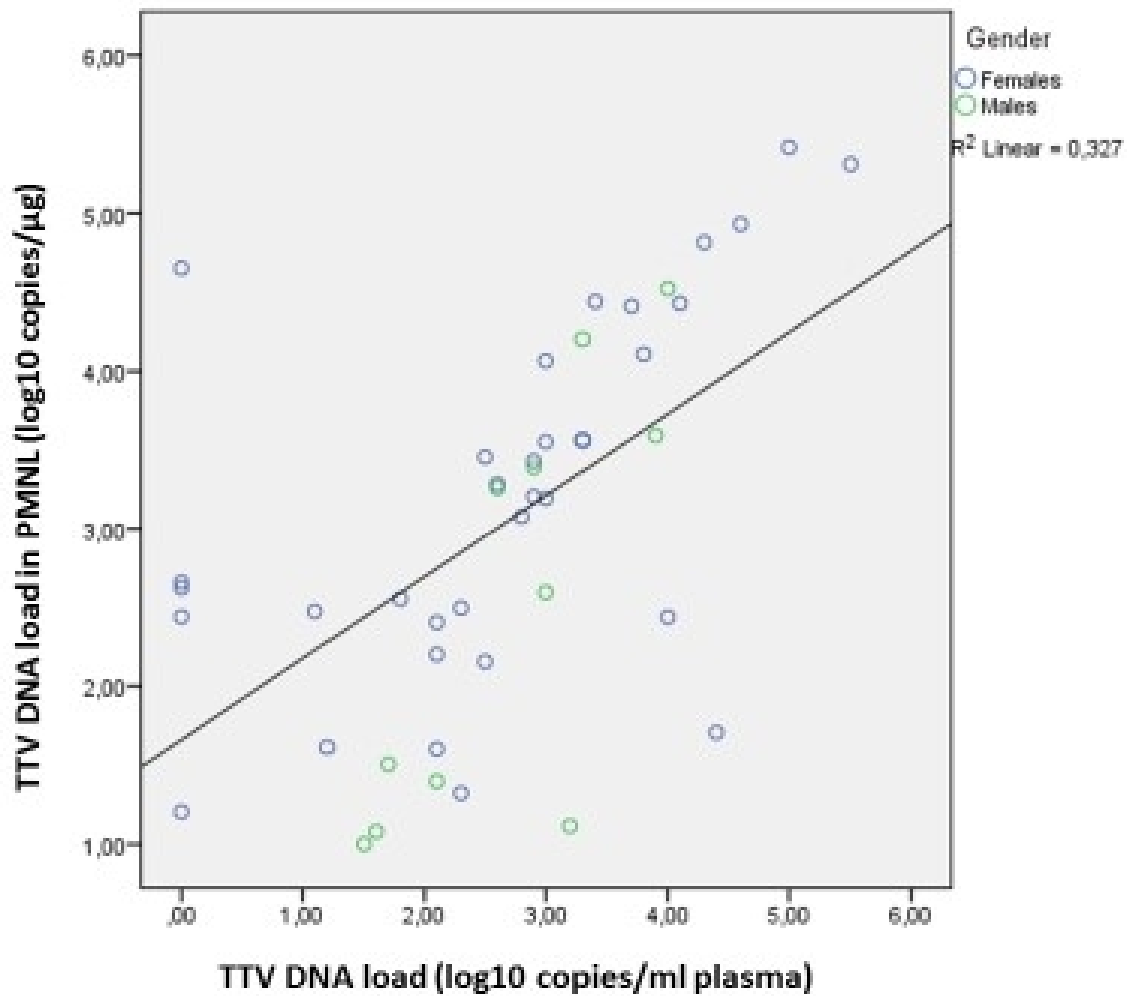
**Ln Neutrophiles**

**Ln Lymphocytes**

No Significant correlations between TTV DNA loads (log-transformed data) and neutrophils or lymphocytes (Ln-transformed data) (R=-0.054, P=0.376; R=0.019, P=0.751) were found. Viral loads were expressed as number of viral genomic copies per µg of genomic DNA extracted by PMNL (Copies/ µg)

**Fig 7S Pearson correlation between TTV DNA loads in PMNL and TTV DNA viremia in plasma samples from elderly subjects**

Significant positive correlation between TTV DNA loads in PMNL and plasma (log-transformed data) analyzed in 50 elderly subjects (N=50; 36 females and 14 males mean age: 80.4 ± 9.7) (R=0.571, P<0.0001). 6/50 (12%) resulted TTV negative plasma samples. Viral loads were expressed as number of viral genomic copies per µg of genomic DNA extracted by PMNL (Copies/ µg) or per ml of plasma.