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## **Prevalence and loads of torquetenovirus (TTV) in the European MARK-AGE Study population**

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## **Abstract**

Torquetenovirus (TTV) viremia has been associated with increased mortality risk in the elderly population. This work aims to investigate TTV viremia as a potential biomarker of immunosenescence. We compared levels of circulating TTV in 1,813 participants of the MARK-AGE project, including human models of delayed (offspring of centenarians - GO) and premature (Down syndrome - DS) immunosenescence. The TTV load was positively associated with age, CMV antibody levels and the Cu/Zn ratio, and negatively associated with platelets, total cholesterol and total IgM. TTV viremia was highest in DS and lowest in GO, with intermediate levels in the SGO (spouses of GO) and RASIG (randomly recruited age-stratified individuals from the general population) populations.

In the RASIG population, TTV DNA loads showed a slight negative association with CD3+T-cells and CD4+T-cells. Finally, males with  $\geq 4\log$  TTV copies/ml had a higher risk of having a CD4/CD8 ratio  $< 1$  than those with lower viremia (OR = 2.85, 95%CI: 1.06-7.62), as well as reduced CD3+ and CD4+T-cells compared to males with lower replication rates ( $< 4\log$ ), even after adjusting for CMV infection.

In summary, differences in immune system preservation are reflected in the models of delayed and premature immunosenescence, displaying the best and worst control over TTV replication, respectively. In the general population TTV loads were negatively associated with CD4+ cell counts, with an increased predisposition for an inverted CD4/CD8 ratio for individuals with TTV loads  $\geq 4\log$  copies/ml, thus promoting an immune risk phenotype.

**Keywords:** TTV, aging, immunosenescence, CD4/CD8 ratio, Down syndrome

## Introduction

Torquetenovirus (TTV) is a highly prevalent, nonpathogenic circular single-stranded DNA virus and represents the most abundant component of the human virome (1). Its circulating levels may be related to the status of the immune system, as TTV load increases with age and is associated with a decreased natural killer activity as well as with an increased risk of mortality in the elderly population (2). Although it is not yet clear whether TTV can play a direct pathogenic role, the virus might aggravate the course of some diseases (3–5) through an increased stimulation of the inflammatory response (6).

Although the TTV DNA plasma load has been positively associated with Cytomegalovirus (CMV) IgG serostatus (2,7), it has been reported as an independent risk factor of mortality in the elderly (2). During aging persistent subclinical CMV infection causes immune perturbations characterized by an accumulation of memory T lymphocytes and a reduction of naïve T-cells that may hamper immunity to new pathogens. This phenomenon may accelerate immunosenescence, (8–10) leading to persistent systemic inflammation, and finally to increased morbidity and mortality (11–14). Longitudinal studies in octogenarian and nonagenarian subjects have demonstrated an immune risk profile (IRP) predictive of increased 2-year mortality. The IRP is associated with persistent CMV infection and characterized by high percentages of CD8+ and low percentages of CD19+ and CD4+ cells, with an inverted CD4/CD8 ratio (15–18).

Analogously to aged people, also Down syndrome (DS) subjects are characterized by immune dysregulation, with alterations in adaptive immunity, lack of diversity among naive cells typical of immunosenescence, decreased number of circulating B cells and impaired humoral immune response after vaccination (19, 20). By contrast, in the offspring of

centenarians the adaptive immune system (both B- and T-cell arms) appears youthful and better preserved, favoring resistance to infections (21,22). However, TTV prevalence in DS and offspring of centenarians and its influence in B-cells, T-cell subsets and CD4/CD8 ratio are as yet unknown.

The purpose of the present study is to measure TTV loads in GO (offspring of centenarians), elderly controls (**R**andomly recruited **A**ge-**S**tratifed **I**ndividuals from the **G**eneral population: RASIG and **S**pouses of **G**O: SGO) and DS subjects recruited in the MARK-AGE project and to evaluate the impact of TTV infection on immune T-cell subsets.

## **METHODS**

### **Study population, recruitment, data and blood collection**

MARK-AGE is a Europe-wide cross-sectional population study aimed at identifying biomarkers of aging (23).

In the present study, we measured TTV loads in peripheral blood from 1,813 participants in the age range of 35–75 years recruited in eight different European countries. The population studied consisted of 1,441 RASIG (Randomly recruited Age-Stratified Individuals from the General population) (mean age  $55.38 \pm 0.30$ ), 226 GO (GEHA offspring) (mean age  $63.99 \pm 0.77$ ), 123 SGO (spouses of GEHA offspring) (mean age  $65.08 \pm 1.05$ ) and 23 DS (Down syndrome) (mean age  $41.43 \pm 2.44$ ). GO comprised subjects born from a long-living parent belonging to a family with long-living sibling(s), such as the “90+ sib-pairs” recruited within the framework of the EU Integrated Project GEHA, and designated GEHA offspring (GO).

Subjects were randomly selected from the whole MARK-AGE population based on an estimation of the sample size (see supplementary data section “estimation of sample size”). Seropositivity for HIV, HBV (except seropositivity by vaccination) and HCV represented

exclusion criteria. Details of the recruitment procedures and the collection of anthropometric, clinical and demographic data, as well as of laboratory parameters assays have already been reported (24–26).

Anticoagulated whole blood, obtained by phlebotomy after overnight fasting, was collected. Prepared samples of plasma, peripheral blood mononuclear cells (PBMC) and whole blood from the various recruitment centers were shipped to the MARK-AGE Biobank located at the University of Hohenheim, Stuttgart, Germany. From the Biobank, coded samples were subsequently sent to the Scientific and Technological Pole of INRCA of Ancona, Italy, on dry ice, where they were stored at -80°C until use.

### **Biomarker assessment**

Using the biological samples (plasma, serum, PBMC), an extensive set of biomarkers was measured such as laboratory parameters, biochemical, immunological, and oxidative stress biomarkers. The complete list of biomarkers has already been described (23,26).

### **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. The presence and load of TTV DNA were determined in a single step in-house TaqMan PCR assay as described elsewhere (27). This assay is called “universal PCR” because it uses forward and reverse primers designed on a highly conserved segment of the untranslated region of the viral genome, and it quantifies the total load of TTV DNA amplifying, without discrimination between the TTV species that circulate in the blood of a single subject.

The lower limit of detection was 10 copies of TTV DNA per ml of blood. The procedures used for copy number quantification and the assessment of specificity, sensitivity, intra- and



inter-assay precision and reproducibility have already been described (27). The detection of anti-TTV antibody titer was not performed, since quantitative serology assays for TTV are not commercially available.

### **Cell phenotyping**

Lymphocyte subsets in PBMC samples from MARK-AGE biobank were analyzed by FACS as described previously (28). Briefly, we used the BD Multitest™ IMK kit (340503) to identify and determine the percentage and absolute counts of the following mature human lymphocyte subsets in PBMC: T lymphocytes (CD3+), B lymphocytes (CD19+), T lymphocytes (CD3+CD4+ and, CD3+CD8+) and natural killer (NK) lymphocytes (CD3-CD16+ and/or CD56. Samples were recorded and analysed on the FACS Calibur (Beckton-Dickinson, Warsaw, Poland) using the Cell-Quest software (BD) and Multiset software (Beckton-Dickinson), respectively.

### **CMV IgG antibody titer**

CMV-specific IgG levels 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 as positive.

### **Statistical analysis**

Subject characteristics were reported as mean  $\pm$  standard error of the mean (SEM) or percentages for continuous and categorical variables, respectively. For continuous variables, the normal distribution was verified by the 1-sample Kolmogorov–Smirnov test. All the variables not normally distributed were log-transformed. Differences among groups were checked by the One-way Analysis of Variance for continuous variables and Pearson's  $\chi^2$  test

for categorical variables. To assess the sample size needed to detect a minimum difference of 0.25 in the log TTV copies with a statistical power of 0.90, G\*Power software was used.

ANOVA (after correction for several confounding factors) was used to evaluate differences in TTV DNA loads among DS, GO, SGO and RASIG or other categorical variables. A linear regression analysis using enter and stepwise methods was carried out to explore the main predictors of TTV DNA loads. The variables inserted were: TTV DNA loads, age, gender, country, body mass index (BMI), hemoglobin, red blood cells, leucocyte count, neutrophils, monocytes, lymphocytes, platelets, C-reactive protein (CRP), ceruloplasmin, fibrinogen, homocysteine, albumin, Cu/Zn ratio, iron and selenium plasma levels, lipid profile, creatinine, fasting glucose, Hemoglobin A1c, CMV and hepatitis B virus (HBV) IgG antibody titers, as well as total immunoglobulin titers. Linear regression was also performed to search for an association between TTV DNA loads and lymphocyte subsets in the RASIG population. A multivariable binary logistic regression, stratifying for gender, was used to evaluate the markers independently associated with a CD4/CD8 ratio <1 (dependent variable). The following variables were included in the analyses: age, countries, neutrophils, lymphocytes, monocytes, TTV copies  $\geq 4$  log (binary variable), CMV IgG antibody titer, albumin, CRP, lipid profile, Cu/Zn ratio.

All the analyses were performed using the SPSS/Win program (version 22.0; Spss Inc., Chicago, IL).

## **Results**

### **Characteristics of the MARK-AGE population**

Table 1 reports the main characteristics of the RASIG, GO and SGO populations, subdivided by three classes of TTV loads: TTV negative, <4 log copies/ml blood, and  $\geq 4$  log copies/ml

blood. The TTV DNA cut-off value of 4 log copies/ml of blood corresponds to 3.8 log copies/ $\mu$ g of genomic DNA from whole blood samples and is comparable to the cut-off value found associated with an increased risk of mortality in elderly subjects (4 log copies/ $\mu$ g of genomic DNA from polymorphonuclear leukocytes) (2).

Subjects with  $\geq 4$  log TTV copies/ml blood were older than the other groups and the TTV negative class had a higher percentage of females than males. However, no differences were found in the examined laboratory parameters such as red blood cell number, hemoglobin, white blood cell count, number of neutrophils, lymphocytes, monocytes and platelets, albumin, CRP, fibrinogen, lipid serum levels, fasting glucose, hemoglobin A1c (HbA1c), creatinine, BUN, and CMV IgG antibody titer.

Characteristics of the DS group, including lymphocytes subsets, broken down by the three aforementioned classes of TTV loads are reported in table 1S (Supplementary material). No differences were observed according to TTV viremia groups.

Interestingly, TTV prevalence differed between European countries. In particular, the percentages of subjects with TTV viremia  $\geq 4$  log were higher in Poland, Greece, Italy and Finland than in other countries, with Germany, Belgium, the Netherlands and Austria having the lowest TTV prevalence (Pearson  $\chi^2$ ;  $p < 0.00001$  Table 3S, Supplementary material).

### **Main predictors of TTV DNA loads in the RASIG population and DS subjects**

Independent biomarkers associated with TTV DNA loads (dependent variable) were identified by a linear regression analysis using enter (Table 2) and stepwise methods (Table 2S, Supplementary material). TTV DNA levels were positively associated with age, CMV antibody levels and Cu/Zn ratio. Moreover, a higher TTV replication was observed in males compared to females, and TTV loads changed according to countries (the latter is also shown

in Table 3S, Supplementary material). A negative association was found with platelets, total cholesterol and total IgM and only a trend was observed for serum creatinine levels. No association was found with BMI, hemoglobin, red blood cells, neutrophils, lymphocytes, CRP, ceruloplasmin, fibrinogen, homocysteine, albumin, free fatty acids, triglycerides, HDL, glucose, HbA1c, HBV IgG antibody titer, IgA, IgE, IgG total titers, Se or Fe plasma levels. Predictors of TTV DNA loads found in RASIG were analyzed in DS subjects by means of a Generalized linear model. No significant associations were found for age, gender, Cu/Zn ratio, CMV IgG titer, platelets and total cholesterol, while the positive association between IgM levels and TTV loads was also found in DS (Table 4S, Supplementary material).

### **TTV DNA loads in DS, GO, SGO and RASIG participants**

TTV levels were higher in DS persons compared to GO, SGO and RASIG subjects (Fig.1,  $p<0.001$ ), while the GO group showed the lowest TTV viremia ( $p<0.05$ ). No difference was present in the distribution of subject groups according to TTV classes (TTV negative,  $TTV < 4 \log$ ;  $TTV \geq 4 \log$ ) as reported in Table 5S (Supplementary material).

### **TTV DNA loads and lymphocyte subsets in the RASIG population and DS**

In a subgroup of 940 RASIG (488 females: mean age,  $55 \pm 13$ ; 452 males, mean age,  $55 \pm 12$ ) a linear regression correcting for age gender and country evidenced a slight negative association between TTV DNA loads, CD3+T-cells (Beta=-0.129,  $p<0.0001$ ), and CD4+T-cells (Beta= -0.104,  $p<0.01$ ) (Fig. 2A, 2B) as well as a positive association with CD16+CD45+CD56+ NK cells (Beta= 0.072,  $p<0.05$ ) (Fig 2D). Significant associations among TTV viremia, CD3+T-cells, CD4+ T helper and NK cells were also confirmed in the whole population (Tables 7S, 8S, 10S, Supplementary material). No significant association was found among TTV viremia and CD8+ T-cells or B cells in both RASIG (Fig 2C, Table 6S, Fig1S, Supplementary material) and the whole population (Table 9S, Table 11S,

Supplementary material). In DS subjects no significant differences were observed for lymphocyte subsets and CD4/CD8 ratio according to TTV classes (TTV negative;  $<4\log$  TTV copies/ml blood;  $\geq 4\log$  TTV copies/ml blood) (Table 1S, Supplementary material). A lack of significance might be due to the limited number of data available for the DS cohort (n.19).

### **Biomarkers associated with a CD4/CD8 Ratio $<1$**

We performed a binary logistic regression (Table 3) to evaluate the markers independently associated with a CD4/CD8 ratio  $<1$  (as a dependent variable). Since an inverted CD4/CD8 ratio is more common in males than in females (15), and since it is more prevalent after the middle age (17), the analysis was stratified according to gender in subjects over 54 years old. Cu/Zn was the sole factor associated with an inverted CD4/CD8 ratio in females, while neutrophil and lymphocyte counts, as well as  $\geq 4 \log$  TTV copies/ml blood were associated with CD4/CD8 ratio  $<1$  in males. In particular, men with  $\geq 4 \log$  TTV copies/ml blood had a 2.8 times higher risk of having a CD4/CD8 ratio  $<1$  than those with a viral load  $< 4 \log$  (OR = 2.85, 95%CI: 1.06-7.62). Although CMV-IgG positive individuals showed a lower CD4/CD8 ratio (15), the CMV antibody titer was not associated with a CD4/CD8 ratio  $<1$  either in males or in females. However, the CMV antibody titer was negatively associated with the CD4/CD8 ratio in a linear regression model adjusted for age, gender and countries in the whole MARK-AGE population (age range 35-75 years) (Beta=-0.196  $p<0.0001$ ) (Table 12S Supplementary material).

### **TTV/CMV positivity classes in the MARK-AGE population**

The distribution of TTV/CMV positivity classes (CMV-TTV-; CMV+TTV-; TTV+CMV-; TTV+CMV+) is reported in the Supplementary Table 13S. We found no difference among RASIG, GO and SGO subjects. Moreover, there were no differences in the distribution of the

virus positivity classes according to the CD4/CD8 ratio  $<1$  or  $>1$  in the RASIG population in both genders (Table 14S, Supplementary material).

## **Discussion**

Age-associated changes in immune functions, collectively termed immunosenescence, are characterized by a diminished adaptive immune competence and an increased low-grade chronic pro-inflammatory status (29). Chronic infections such as CMV play a role by acting as activators of T-lymphocytes and contributing to inflammation and tissue damage (30,31). Longitudinal studies in healthy donors aged over 80-90 years old demonstrated an IRP – characterized by a latent CMV infection, an inversion of the CD4/CD8 T-cell ratio, and T-cell subset accumulation which was predictive of mortality (17,18). However, in another cohort of subjects over 80 years old in Belgium, it was found that elderly CMV-negative women with a CD4/CD8 ratio  $> 5$  experienced the highest mortality rates, independent of age and comorbidity (32). This suggests a different clinical impact of the CD4/CD8 ratio and CMV infection, that may depend on differences in genetic background, lifestyle, environmental factors and age range. Moreover, it is still uncertain whether CMV-associated changes in the immune system are compensatory and beneficial or pathological and detrimental for the immune system and longevity (33).

Some evidence suggests that CMV is only partially responsible for the induction of immunosenescence, since CMV seronegative elderly subjects show age-related changes in the function and composition of the immune cells (8). TTV is a human DNA virus that causes asymptomatic viremia, with a high prevalence in the general population, and it has been suggested as a surrogate marker of immunosuppression (34,35). Recently, an association of TTV load with reduced NK cell activity has been found (2), but the implication of TTV in immunosenescence and inverted CD4/CD8 ratios in aging has not yet been investigated.

In the present paper, we reported the virus prevalence in several European countries. A relatively high rate (from 84 to 88%) of TTV infection was observed in Italy, Poland, Greece and Finland. These data are consistent with the known high TTV prevalence in healthy adult populations, around 70 to 90 % (2,7,36). We demonstrated for the first time a higher increment of TTV loads in DS subjects compared to GO, SGO and RASIG subjects, which might depend on an accelerated immunosenescence condition associated with DS (37). DS is a genetic disorder caused by a partial or complete trisomy of chromosome 21, also characterized by accelerated aging (38) and immunological abnormalities (39) that may cause increased susceptibility to bacterial and viral infections (40,41). DS patients have a decreased number of circulating T and B cells (40), with reduced levels of CD4+ naive cells and increased CD8+ T lymphocytes, compared to healthy subjects (37,42,43). In this context, the increased TTV replication rate in DS might also play a role in T-cell subset abnormalities. However, our data show that lymphocyte subsets were similar among TTV DNA load classes in DS subjects. This fact may depend on the small sample size. Therefore, in order to demonstrate the influence of TTV on T-cell subset in DS, further studies on a larger cohort of DS patients are necessary. By contrast, GO showed the lowest TTV loads that are likely related to their preserved immune function (21). Indeed, studies in GO have reported that their T-cells show fewer signs of immunosenescence than those from age-matched controls, including the increased presence of naive T-cells and reduced numbers of senescent CD8 cells (21). Moreover, further evidence established an association between higher numbers of naïve, activated/memory and effector/memory T-cells and 5-year survival rate in centenarians (44). Interestingly, males with  $\geq 4$  log TTV copies/ml blood showed a more frequent inverted CD4/CD8 ratio than those with lower viral loads. The association of higher TTV viral loads with an inverted CD4/CD8 ratio might in part explain the increased risk of mortality (17), previously observed in an Italian elderly population (2). The lack of this

association in women could depend on several reasons such as the lower TTV replication here observed in females, the reduced percentage of women with an inverted CD4/CD8 ratio (29% vs 71% in men), in line with other studies (17,28), and the “relatively young” age (i.e. under 75 years old) of the population examined here. It has been shown that the prevalence of CD4/CD8<1 increases significantly above the age of 60 with further increments in octa-nagenarians, while it is lower in females than in males throughout all stages of life (15–17). In the RASIG population, we also observed a slight negative association between TTV DNA loads and CD3+T or CD4+T cells, which became more evident in subjects over 68 years old (Fig2S Supplementary material). This result reinforces the hypothesis that TTV load is more associated with immunological changes in the elderly population, thus being an optimal candidate as a marker of immunosenescence.

Noteworthy, CD3+ and CD4+ T-cells percentages were significantly reduced in RASIG males with  $\geq 4\log$  TTV copies/ml blood compared to males with lower replication rates ( $< 4\log$ ), even after adjusting for CMV seropositivity (Fig 3S, Supplementary material).

Long-lived individuals display preserved phenotypic and functional characteristics of NK cells. Our current findings show that the higher number of NK cells was related to increased TTV viral loads, more clearly in RASIG over 68 years old (Fig 2S, C Supplementary material), probably to compensate for the reduced NK cell functionality (2), similarly to what occurs in DS (45).

In the present cohort, we confirmed the positive association of TTV viral load with age and CMV antibody titer (2). However, in DS subjects we did not find a significant association between TTV replication rate and age, probably due to the low number of subjects. A relation between TTV and CMV infections was also observed in transplant patients, where TTV viremia was associated with CMV reactivation (46).



The negative association between TTV loads and platelet count in our population has also been reported in patients with hepatitis C virus-related chronic liver disease, pointing to the role of some TTV genotypes, including genotype 1, in aggravating the thrombocytopenia (47). Furthermore, the inverse relation between TTV and serum cholesterol levels could suggest a dysregulation of lipid metabolism as also observed in other chronic virus infections (48). Noteworthy, TTV viremia was positively correlated with the Cu/Zn ratio, suggesting an increased subclinical immune-inflammatory status (49,50) which cannot be detected by other systemic inflammatory markers (CRP ceruloplasmin, fibrinogen, homocysteine). This result may eventually support the proinflammatory role of the virus previously observed “in vitro” (6). Although TTV viremia did not affect the B-cell count, it was associated with a decreased IgM total titer in both RASIG and DS. This association suggests an impaired functionality of B-cells, as also observed during aging or in DS subjects (51,52). On the other hand, GO subjects have more abundant naïve B cells and higher IgM levels which are likely to contribute to their increased resistance to new infections and their prolonged survival (44,53). Further research is needed to better understand if TTV infection is responsible for a decreased B cell function and an impaired IgM production.

In summary, although the lack of a baseline sample makes it impossible to distinguish between acute and chronic TTV infections, TTV viremia increased in DS subjects, a human model of accelerated immunosenescence, while it was reduced in GO, a population characterized by a preserved immune system. TTV replication was negatively associated with CD4<sup>+</sup> cell counts. Moreover,  $\geq 4\log$  TTV copies/ml blood could predispose to an inverted CD4/CD8 ratio in males promoting an immunosenescent profile, increasing the risk of mortality in elderly subjects. Further studies are necessary to clarify if the TTV replication level represents an indicator of immune dysfunction or if the virus may play a causal role in immunosenescence promoting the accumulation of pro- inflammatory and senescent T-cells.

Besides, since it is well documented that the majority of people harbor multiple TTV species in the circulation, it is necessary to characterize the exact relation between co-infections by two or more of such species and the range of viral loads.

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

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**Table 1. Characteristics of subjects selected from the whole MARK-AGE population for TTV analysis**

	<b>TTV negative n.447</b>	<b>TTV copies&lt;4log n.627</b>	<b>TTV copies ≥4log n.716</b>	<b>P value</b>
Age (yrs)	59.3±1.0	59.9±0.9	63.2±0.8	0.005
Females (%)	272 (60.9)	332 (53.0)	336 (46.9)	0.0002
RBC (x10 <sup>6</sup> /μl)	4.74±0.07	4.74±0.05	4.80±0.05	NS
Hemoglobine (g/dl)	14.27±0.19	14.21±0.16	14.39±0.15	NS
WBC(x10 <sup>3</sup> /μl)	6.38±23.31	18.2±20.79	9.1±19.40	NS
Neutrophils (x10 <sup>3</sup> /μl)	3.58±14.67	10.50±13.08	5.73±12.16	NS
Lymphocytes(x10 <sup>3</sup> /μl)	1.93±4.18	4.14±3.72	2.70±3.46	NS
Monocytes (x10 <sup>3</sup> /μl)	0.51±1.38	1.00±1.19	0.87±1.11	NS
Platelets (x10 <sup>3</sup> /μl)	233.0±5.1	230.6±4.5	233.0±4.2	NS
Albumin (g/dl)	4.04±0.03	4.10±0.02	4.04±0.02	NS
CRP (μg/L)	2.59±0.29	2.18±0.26	2.56±0.25	NS
TC (mmol/L)	5.48±0.09	5.69±0.08	5.54±0.08	NS
HDL (mmol/L)	1.48±0.03	1.51±0.03	1.49±0.03	NS
LDL (mmol/L)	3.29±0.08	3.44±0.07	3.30±0.07	NS
TG(mmol/L)	1.39±0.07	1.28±0.06	1.27±0.06	NS
Fibrinogen (mg/mL)	3.83±0.13	3.93±0.11	3.90±0.10	NS
FG (mmol/L)	5.12±0.09	5.09±0.08	5.07±0.07	NS
HbA1c (%)	5.97±0.05	5.96±0.05	5.99±0.04	NS
BUN (mmol/L)	5.81±0.09	5.79±0.06	5.89±0.06	NS
Creatinine (μmol/L)	74.1±1.2	75.5±1.0	73.8±0.9	NS
CMV (U/L)	36.06±3.75	40.44±3.33	44.72±3.19	NS

Data are reported as mean ± Standard Error of the Mean (SEM)

The laboratory parameter analysis was adjusted for age, gender, countries and class of subjects (GO, SGO, RASIG)

RBC Red Blood Cells, WBC white blood cells, CRP C-reactive protein, TG triglycerides, TC total cholesterol, HDL high-density lipoprotein cholesterol; LDL Low- density lipoprotein cholesterol, FG fasting glucose, HbA1c Hemoglobin A1c, BUN blood urea nitrogen, CMV Cytomegalovirus antibodies

**Table 2. Multivariate linear regression analysis for variables independently associated with TTV DNA loads in the RASIG population**

Predictors	Unstandardized Coefficients		Standardized Beta Coefficients	P value
	B	Std. Error		
Age	0.019	0.003	0.214	<b>0.000</b>
Gender	0.246	0.111	0.115	<b>0.027</b>
Countries	0.039	0.020	0.076	<b>0.047</b>
BMI	-0.027	0.054	-0.019	0.614
Hemoglobine	0.120	0.467	0.011	0.798
RBC	-0.348	0.226	-0.054	0.124
Neutrophils	-0.054	0.102	-0.027	0.596
Lymphocytes	-0.078	0.130	-0.034	0.547
Monocytes	0.164	0.131	0.079	0.210
Platelets	-0.285	0.116	-0.086	<b>0.014</b>
CRP	0.043	0.042	0.039	0.313
Ceruloplasmin	-0.149	0.189	-0.034	0.432
Fibrinogen	0.057	0.116	0.019	0.622
Homocysteine	-0.048	0.110	-0.017	0.665
Cu/Zn	0.424	0.211	0.095	<b>0.045</b>
Albumin	0.612	0.568	0.043	0.282
FFA	0.048	0.082	0.020	0.560
TG	-0.089	0.100	-0.041	0.374
TC	-0.705	0.243	-0.120	<b>0.004</b>
HDL	0.069	0.190	0.018	0.716
FG	0.020	0.276	0.003	0.942
HbA1c	0.023	0.492	0.002	0.963
Creatinine	-0.421	0.227	-0.082	0.065
CMV IgG titer	0.072	0.025	0.102	<b>0.004</b>
HBV IgG titer	-0.001	0.023	0.002	0.999
IgA	0.005	0.086	0.002	0.957
IgE	0.032	0.025	0.043	0.205
IgG	0.146	0.169	0.031	0.386
IgM	-0.329	0.072	-0.162	<b>0.000</b>
Se	-0.102	0.222	-0.017	0.645
Fe	0.022	0.099	0.008	0.825

Gender was categorized as follows: 1=males and 0=females

RBC Red Blood Cells, CRP C-reactive protein, Cu/Zn plasma Copper/Zinc ratio, FFA plasma free fatty acid, TG triglycerides, TC total cholesterol, HDL high-density lipoprotein cholesterol; LDL Low- density lipoprotein cholesterol, FG fasting glucose, HbA1c Hemoglobin A1c, BUN blood urea nitrogen, CMV Cytomegalovirus antibodies, HBV Hepatitis B virus antibodies, IgA Immunoglobulin A titer, IgE Immunoglobulin E titer, IgG Immunoglobulin G titer, IgM Immunoglobulin M titer, Se plasma selenium, Fe plasma iron.

**Table 3. Determinants associated with CD4:CD8 ratio lower than 1 in Males and Females aged more than 54 years**

	Females (N=388)			Males (N=348)		
	OR	95 % CI	P value	OR	95 % CI	P value
Countries	1.171	0.826-1.661	0.375	0.917	0.734-1.146	0.446
Age	1.079	0.964-1.209	0.186	1.020	0.941-1.106	0.624
Neutrophils	0.672	0.267-1.697	0.401	0.581	0.349-.969	<b>0.037</b>
Lymphocytes	1.001	1.000-1.003	0.176	1.001	1.000-1.002	<b>0.013</b>
Monocytes	0.999	0.992-1.007	0.838	0.999	0.995-1.002	0.395
TTV copies $\geq 4\log^*$	1.056	0.246-4.540	0.941	2.850	1.066-7.622	<b>0.037</b>
CMV IgG titer	1.006	0.990-1.022	0.446	1.009	0.999-1.019	0.077
HBV IgG titer	0.975	0.470-2.023	0.946	0.842	0.185-3.842	0.824
Albumin	1.208	0.924-1.580	0.167	0.897	0.764-1.052	0.182
CRP	1.026	0.797-1.321	0.842	0.773	0.564-1.058	0.108
TC	1.169	0.226-6.037	0.852	0.657	0.210-2.052	0.470
TG	1.773	0.754-4.168	0.189	1.070	0.638-1.796	0.797
LDL	0.901	0.135-6.023	0.914	1.831	0.447-7.499	0.400
Cu/Zn ratio	5.211	1.051-25.846	<b>0.043</b>	1.764	0.367-8.484	0.479

\* using, as reference group, TTV < 4log

### **Figure 1. TTV DNA loads in peripheral blood from GO, SGO RASIG and DS participants**

DS showed significantly higher TTV DNA loads vs. all the other classes of subjects, GO participants displayed the lowest viral loads. ANCOVA analysis correcting for age, countries and gender was applied; data are reported from the model adjusted mean  $\pm$  Standard Error of the Mean (SEM); \* $p < 0.05$  as compared to RASIG and SGO; \*\* $p < 0.001$  as compared to RASIG, GO and SGO

### **Figure 2. Linear regression between TTV DNA loads and lymphocyte subsets in the RASIG population**

A negative association between CD3+T-cells (Beta=-0.139,  $p < 0.0001$ ), and CD4+T-cells (Beta= -0.115,  $p < 0.001$ ) and TTV DNA loads (log-transformed data), after adjusting for age, gender and countries (Fig. 2A, 2B). A positive association were observed between CD16+CD45+CD56+ NK cells (Ln-transformed data) and TTV DNA loads (log-transformed data) (Beta= 0.089,  $p < 0.01$ ) after adjusting for age, gender and country (Fig. 2D). Lymphocyte subsets were analyzed in a subgroup of 940 RASIG (488 females: mean age,  $55 \pm 13$ ; 452 males: mean age,  $55 \pm 12$ ) (Fig. 2C).

Fig1\_HR

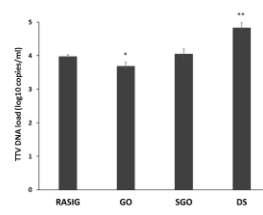




Fig2\_HR

