

Association between TTV viremia, chronic inflammation, and ischemic heart disease risk: Insights from MARK-AGE and Report-Age projects

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Abstract

The implication of Torquetenovirus (TTV) in Ischemic Heart Disease (IHD) has not been thoroughly explored. This study investigated the association between TTV viremia, proinflammatory cytokines, and IHD risk in an aging population. This cross-sectional study included 900 non-IHD subjects (NIHD) and 86 individuals with IHD (aged 55 to 75 years) selected from the MARK-AGE project. Results were verified in another independent Report-Age cohort, including 94 inpatients with chronic IHD and 111 inpatients with no evidence of IHD (NIHD) (aged 65 to 96 years). Multivariable logistic regression in the MARK-AGE cohort revealed that male sex, TTV viremia $\geq 4\log$, Cu/Zn ratio, diabetes, hypertension and smoking were significant IHD predictors. Notably, TTV viremia $\geq 4\log$ independently increased the IHD risk (OR: 2.51, 95% CI: 1.42-4.43), confirmed in the Report-Age cohort (OR: 4.90, 95% CI: 2.32-10.39). In a RASIG subgroup, individuals with TTV viremia $\geq 4\log$, both with and without IHD, exhibited increased plasma pro-inflammatory cytokine levels (IFN- γ , IL-1 β , IL-6, IL-10, IL-12p70, TNF- α) compared to those with TTV viremia $< 4\log$. No significant difference in cytokine production was observed between IHD patients and NIHD with TTV viremia $\geq 4\log$. A positive correlation between TTV viremia and DNA methylation estimator of leukocyte telomere length was observed in Report-Age patients. Additionally, IHD Report-Age patients with TTV viremia $\geq 4\log$ displayed higher NLR and SIRI index than those with TTV viremia $< 4\log$.

In conclusion, a high TTV viremia is associated with an elevated IHD risk in the older population, potentially arising from an augmented proinflammatory response and immunosenescence.

Keywords: Torquetenovirus, Ischemic Heart Disease, aging, inflammation

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Introduction

Ischemic Heart Disease (IHD) is a chronic inflammatory disease characterized by atherosclerosis of the coronary arteries and a serious health threat worldwide, particularly in the older population (1). The severity of IHD is associated with cardiovascular death and all-cause mortality (2). Plentiful evidence establishes a link between chronic viral infections and the development of IHD. For instance, an elevated prevalence of coronary artery disease has been observed in patients with chronic hepatitis C or human immunodeficiency virus (3,4). Moreover, cytomegalovirus (CMV), which induces chronic adaptive immune activation and advances immunosenescence (5), might accelerate atherogenesis; CMV viremia has also been linked to acute coronary syndrome (6). One potential mechanism by which viruses may contribute to the development of atherosclerosis and cardiovascular disease is through the induction of cellular senescence (CS). CS is characterized by increased β -galactosidase activity and elevated expression of cell cycle arrest markers, such as p16/INK4, p21/WAF-1, and p53. This process is accompanied by the secretion of a senescence-associated secretory phenotype (SASP) and the presence of shorter telomeres (7,8).

Population studies indicate that leukocyte telomere length is associated with a higher incidence of IHD, independent of other risk factors (9,10). Senescent endothelial cells (ECs), vascular smooth muscle cells (VSMCs), foam cells, and macrophages may all play a role in the pathophysiology of atherosclerosis through increased levels of inflammatory cytokines and adhesion molecules and reduced nitric oxide production (8).

Multiple studies have demonstrated that various viral infections, including CMV (11,12) can trigger CS responses. Additionally, in HIV patients, the activation and senescence biomarkers of T cells have been found to correlate positively with the presence of subclinical atherosclerosis, independent of age, sex, and traditional cardiovascular risk factors (13).

Increasing evidence points to the involvement of Torquetenovirus (TTV) in immunosenescence among the older population (14,15). TTV is a small, non-enveloped, single-stranded DNA virus belonging to the Anelloviridae family with a high prevalence in the general population (14,16). TTV constitutes a major component of the human virome [9]. Although its exact role and clinical significance are still unclear, TTV DNA levels can be used to determine the degree of immunosuppression (17,18). Previously, our group showed that TTV viremia higher than 4log was associated with immune dysfunction, mortality and an increased risk of frailty in an aging population (14,15,19).

Chronic inflammation and the production of several cytokines are related to the pathophysiology of IHD (20–23), but it is still unclear whether high TTV replication may induce a dysregulation in cytokine production and promote the process of atherogenesis. Moreover, TTV has been found in atheromatous plaque from patients with coronary artery disease (24)

Hence, the goal of this study was to determine the potential relationship between TTV viremia and IHD, along with the production of proinflammatory cytokines and markers of systemic inflammation and DNA methylation estimator of leukocyte telomere length (DNAm-LTL)(25).

Materials and Methods

Study population, recruitment, data and blood collection

This cross-sectional study included 900 healthy control subjects and 86 IHD patients, all of whom were selected from the RASIG (Randomly recruited Age-Stratified Individuals from the General population) participants within the age bracket of 55 to 75 years. These individuals were recruited as part of the European MARK-AGE project (26).

Details of the recruitment procedures and the collection of anthropometric, clinical and demographic data have been previously published (27,28). Health status and IHD diagnosis was evaluated on the basis of detailed questionnaires administered to participants. The self-reported data were supplemented with medical history of previous acute myocardial infarction or myocardial revascularization or angina pectoris (28). Plasma isolation procedure from blood, as well as the shipment and distribution of biological samples have been described (28). Briefly, lithium heparin plasma was prepared from whole blood, obtained by phlebotomy after overnight fasting, and subsequently stored at -80°C . Samples were then shipped from the various recruitment centers to the MARK-AGE Biobank located at the University of Hohenheim, Stuttgart, Germany. From the Biobank, coded samples were subsequently sent to the IRCCS INRCA on dry ice, where they were stored at -80°C until use.

A second cohort of 205 patients, chosen for data validation, was identified from the Report-Age project, adhering to specific criteria such as the availability of blood samples and complete clinical information. The patients were categorized into two groups: 94 individuals with chronic IHD and 111 with other diseases and no evidence of IHD (NIHD). Exclusion criteria encompassed a diagnosis of cancer, acute stroke, sepsis, and acute

pancreatitis. The Report-AGE project, a large-scale observational study of older patients (age >65 years) hospitalized at IRCCS INRCA between June 16, 2012, and November 3, 2017 (29), is registered under Trial Registration no. NCT01397682. Blood samples, collected in EDTA tubes (Becton, Dickinson and Company, New Jersey, USA) within the initial 24 hours following hospital admission, were stored in the INRCA BioGer Biobank. Comprehensive data on the medical history of all participants in this study were retrieved from medical records. Participating physicians and nurses were specifically trained before starting recruitment, as previously described (30,31). Diagnoses were coded in accordance with the International Classification of Diseases, 9th revision (<http://www.icd9data.com/>). The inclusion criterion for chronic IHD was confirmed based on the presence of at least one diagnosis (at admission and/or earlier) with one the following ICD-9 codes: 412 (previous myocardial infarction) 413.x (angina pectoris), 414.0, 414.8, 414.9 (ischemic heart disease). No patients diagnosed with an acute heart attack were present in this cohort (ICD.-9: 410).

Upon hospital admission, laboratory parameters such as blood cell counts, creatinine, albumin, hemoglobin, and aspartate aminotransferase/alanine aminotransferase (AST/ALT) were measured using standardized procedures. The SIRI index was calculated as the formula of neutrophil count * monocyte count/lymphocyte count. GFR was estimated according to Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation(32).

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 (14,33). 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 (33). TTV viremia equal to or exceeding 10000 copies/mL (equivalent to ≥ 4 log copies) was categorized as high-level viremia.

Hs-cTnT and NT-proBNP assays

Serum HS-cTnT was dosed by Elecsys® Troponin T hs STAT assay. Age-specific 99th percentile, normal troponin range levels for HS-cTnT in our laboratory is ≤ 40 pg/ml for over 85 y patients (34). Serum NT-proBNP was assessed on a Dimension Vista® 1500 automated chemistry analyzer using NT-proBNP (PBNP) assays (Siemens Healthineers, Germany) based on LOCI® technology.

Zinc and copper plasma level determinations

Cu/Zn ratio is a relevant biomarker of chronic inflammation associated with aging and a predictor of all-cause mortality in elderly population (35–37). Moreover, elevated serum Cu/Zn-ratio is associated with an increased risk of heart failure and cardiovascular disease (38,39). 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 (37).

Cytokine Assay

BD CBA Human Flex set” and the “BD FACS Array” (BD Biosciences, USA) were used to quantify specifically and simultaneously concentration of the plasma cytokines (IL-1 β , IL-6, IL-10, IL-12p70, TNF- α , and IFN- γ).

Estimation of surrogate DNAm based telomere length in leukocytes (DNAm-LTL)

Genomic DNA was extracted from whole blood using the QIAamp DNA Blood Kit (Qiagen) and bisulfite-converted using the EZ DNA Methylation Kit (Zymo Research). Genome-wide DNA methylation was assessed with the Infinium Human Methylation EPIC BeadChip (Illumina), following the manufacturer's instructions (40). DNAm-LTL Leukocyte telomere length (LTL) estimation (25) was calculated using the R package dnaMethyAge. A user friendly R package to predict epigenetic age and calculate age acceleration from DNA methylation data (<https://github.com/yiluyucheng/dnaMethyAge>).

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 χ^2 test for categorical variables. ANOVA, after correction for age, sex (Report-Age cohort) or age, sex and country (RASIG group) was used to evaluate differences in inflammatory markers between TTV DNA viremia categories among NIHD, IHD and NIHD groups. Multi-logistic regression analysis was carried out to explore the association between the TTV and the presence or absence of IHD in RASIG participants. The variables included were: age, sex, country, body mass index (BMI), C-reactive protein (CRP), TTV copies $\geq 4\log$ (binary variable), Cu/Zn ratio, CMV IgG antibody levels, diabetes, hypertension, hypercholesterolemia and smoking. Additionally, a second Multinomial Logistic Regression Analysis was conducted in the Report-Age cohort, incorporating the variables of age, sex, TTV copies $\geq 4\log$ (as a binary variable), Charlson Comorbidity Index, chronic heart failure and the number of medications at discharge. All the analyses were performed using the SPSS/Win program (version 27.0; Spss Inc., Chicago, IL).

Results

Characteristics of the study population

Characteristics of the study population are summarized in Table 1.

IHD patients share a comparable age with the NIHD, but had a higher proportion of males. IHD subjects also displayed elevated BMI, and some inflammatory markers (CRP, Cu/Zn ratio), increased anti-CMV IgG antibody levels, along with reduced levels of total and LDL-cholesterol due to the administration of hypolipidemic drugs, compared to the healthy donors ($p < 0.0001$).

The medications taken by IHD MARK-AGE participants are listed in Table 2S (Supplementary Material). Furthermore, the prevalence of diabetes mellitus, hypertension, and atrial fibrillation was significantly greater in the patient group compared to the NIHD group ($p < 0.001$). 43.0% of IHD subjects had undergone a myocardial infarction in the past. The characteristics of the Report-Age cohort are summarized in Table 1S (Supplementary material). There is no disparity in age, sex distribution, or prevalence of the main comorbidities between IHD and NIHD patients, except for a higher incidence of congestive heart failure in those with IHD and an increased use of beta blockers, vasodilators, statins and antithrombotic agents among IHD patients compared to NIHD patients (Table 1S, Supplementary Material). Additionally, IHD patients exhibited lower eGFR values than NIHD (Table 1S, Supplementary Material) and were predominantly characterized by advanced CKD (G4: 63.7%) and moderate stage CKD (G3b: 36.3%), whereas NIHD patients were more commonly affected by mild to moderate CKD (G3a: 33.3%; G3b: 66.7%).

Multi-logistic regression analysis to determine the association between the TTV and the presence or absence of IHD in the MARK-AGE and the Report-Age cohort

Table 2 shows the results of the association between the participants' sociodemographic and clinical characteristics, TTV, inflammatory markers and IHD, in the adjusted multinomial regression models in the MARK-AGE participants. The main predictors of IHD included male gender, TTV viremia $\geq 4\log$, Cu/Zn ratio, diabetes, hypertension and smoking. TTV viremia $\geq 4\log$ (OR: 2.51, 95% CI: 1.42-4.43) was an independent risk factor of IHD in RASIG participants. The association of TTV viremia $\geq 4\log$ (OR: 4.90, 95% CI: 2.32-10.39) with chronic IHD has been confirmed in the Report-Age cohort adjusting for age, sex, Charlson Comorbidity Index, chronic heart failure and number of medications at discharge (Table 3) even after including the major comorbidities (OR: 5.71 95% CI: 2.72-11.97; Table 3S Supplementary material). TTV viremia was compared among NIHD and IHD patients. IHD patients exhibited significantly higher levels of TTV viremia than NIHD patients (Fig. 1S, Supplementary material).

Plasma cytokine concentrations in IHD and NIHD subjects from MARK-AGE study

Plasma pro-inflammatory cytokine levels were analyzed in a subgroup of 20 IHD patients (7 females and 13 males; mean age 66.4 ± 5.8) and 182 NIHD (110 females and 72 males; mean age 64.6 ± 6.0) from the RASIG cohort. IFN- γ , IL-1 β , IL-6, IL-10, IL-12p70 and TNF- α increased in IHD patients and NIHD with TTV viremia $\geq 4\log$ as compared to those with TTV viremia $< 4\log$ (Fig. 1). Furthermore, IHD who exhibited TTV viremia $\geq 4\log$ had significantly higher plasma cytokine concentrations than NIHD with TTV viremia $< 4\log$. However, their cytokine levels were comparable to those of NIHD with TTV viremia $\geq 4\log$.

indications suggest a link between TTV and inflammation. Indeed, TTV has also been associated with immunosenescence and frailty syndrome (14,15,19), and inflammation has been involved in the pathogenesis of both conditions (43). An elevated TTV DNA load has also been linked to reduced CD4 T-cell counts (14), a condition that is associated with an increased risk of developing cardiovascular events (44). While some evidence suggests a potential role in coronary artery disease for other viruses associated with immunosenescence, such as CMV (6,45), conflicting data also exist (46). In our study, individuals with IHD exhibit higher levels of anti-CMV IgG levels compared to controls in the MARK-AGE population. However, a Multinomial Logistic Regression Analysis reveals no significant association with IHD after adjusting for various confounding factors.

Despite not being classified as a pathogenic virus, TTV has been clinically linked to several chronic immune diseases, including Crohn's disease, where TTV species are more abundant in the relapse rather than remission phase (47). Increased TTV DNA loads have also been associated with pulmonary function and the severity of chronic obstructive pulmonary disease (COPD), in which TTV is highly prevalent and multiple TTV genotype combinations are frequent (48).

We explored the potential association between markers of systemic inflammation, such as the SIRI index and NLR, within our cohorts. No significant differences were noted in the NLR and SIRI parameters or Cu/Zn ratio (Fig. 2S, Supplementary material) among NIHD group, and IHD individuals in the MARK-AGE cohort based on TTV viremia levels. In contrast, IHD patients from the Report-Age cohort exhibited increases in both systemic inflammatory parameters in relation to TTV viremia levels. Although a systemic inflammation, characterized by elevated CRP, Cu/Zn ratio (49), NLR, and SIRI index

parameters, may not be evident in younger individuals with IHD (MARK-AGE cohort) and high TTV viremia, an increased proinflammatory response was observed. Indeed, individuals within the RASIG group with TTV viremia exceeding 4 logs, showed a notable upregulation of several proinflammatory cytokines such as, IFN- γ , IL-1 β , IL-6, IL-10, IL-12p70 and TNF- α . Previous in vitro experiments showed that TTV can induce gene expression of IFN γ , IL6, and IL10 cytokines (50).

All these cytokines may have a significant role in the development and progression of IHD. Interleukins (IL-1 β and IL-6), along with interferon- γ (IFN- γ), and tumor necrosis factor-alpha (TNF- α), are cytokines commonly associated with endothelial dysfunction, vascular inflammation, and the progression of atherosclerosis (51).

TNF- α is a key proinflammatory mediator implicated in various immunopathogenic processes, triggering acute vascular inflammation and facilitating the transition from stable coronary artery disease to acute coronary syndrome by upregulating ICAM-1 (22,51). IL-6 serves as an independent risk factor for cardiovascular mortality and could be a prognostic indicator of cardiovascular events(52). IL-10, typically protective, shows elevated levels in patients with severe coronary stenosis (53,54). Proinflammatory IFN- γ plays a crucial role in regulating the Th1/Th2 ratio and is significantly associated with increased risk and severity of CAD (55,56). IL-12, produced by activated classical antigen presenting cells, is elevated in human atherosclerotic plaques and significantly associated with a higher risk of cardiovascular events and CAD severity(23).

This study is the first to exhibit an association between elevated TTV viremia and increased cytokine production among patients with IHD. Another study, carried out in children, who underwent bone marrow transplantation and experienced liver injury,

revealed an upregulation of IFN- γ , TNF- α , FGF-basic, and MCP-3 in TTV-positive patients when compared to both TTV-negative individuals and the control subjects (57). Further research is necessary to establish the role of TTV in the inflammatory response and its implication in IHD pathogenesis. Another interesting result that might explain the immunosenescent and pro-inflammatory phenotype of subjects with a high TTV DNA load is the negative association found between DNAm-LTL and TTV viremia. DNAm-LTL has been associated with chronological age, sex, ethnicity, lifestyle factors, clinical biomarkers of cardiovascular disease and results to have a better predictive power than LTL for time-to-death and time-to coronary heart disease (25) The correlation between DNAm-LTL and TTV viremia in our study may support the role of viral infection in inducing CS and aligns with other studies that have demonstrated the contribution of virus to accelerated CS (58,59), suggesting another mechanism through which high TTV viremia may be a risk factor for IHD (9,10).

Our study has several limitations that should be acknowledged. In the MARK-AGE cohort, IHD was defined based on self-reported disease diagnosis rather than standardized diagnostic guidelines, which might introduce misclassification bias. In the Report-AGE cohort the diagnosis of IHD was coded according to the International Classification of Diseases, 9th revision that might not be accurate.

However, measurements of high-sensitivity cardiac troponin and NT-proBNP in a subgroup of Report-Age patients showed higher values of both biomarkers in IHD patients compared to NIHD (Fig3S, Supplementary material). Other limitations include the small sample size of IHD patients, the absence of IFN type I and III assays, which are implicated in controlling viremia, the cross-sectional design, that prevents to establish the causal

relationship between TTV and IHD development, the absence of an assessment to determine whether the drugs taken by study participants could impact TTV viremia. Moreover, the lack of a follow-up period to monitor the progression of IHD over time and to evaluate the association of increased TTV viremia and future cardiovascular events. Larger prospective studies should be conducted to examine the diagnostic significance of high TTV viral load in IHD patients.

In conclusion, TTV viral load ≥ 4 log copies/mL of blood were identified as independent IHD risk factor and associated with increased proinflammatory response and reduced DNAm-LTL values.

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Funding:

This work was supported by the European Commission (Project Full Name: European Study to Establish Biomarkers of Human Ageing; Project Acronym: MARK-AGE; Project No: 200880), and by Italian Health Ministry with the PNRR-MAD-2022-12376334 VIROMA project, PROMOTERA project GR-2019-12368606 and Ricerca corrente project.

Conflicts of Interest: The authors declare no conflict of interest.

Acknowledgments:

The authors thank the subjects who participated in MARK-AGE study and BioGer, the IRCCS INRCA Institutional Biobank for providing the samples; a special thanks to the late Oliver Toussaint who dedicated his life to research activity with great energy, creativity and enthusiasm. We remember him with great admiration and affection.

Author Contributions:

Conceptualization, R.G., Methodology, P.G.S., C.M., F.P., F.N., F.D.F; Software, R.G., M.M.V.; BioBank, T.G, Recruitment of Subjects: B.W., (Austrian cohort), F.D.C. (Belgian cohort) J.B. (German cohort), E.S.G. (Greek cohort), C.F., M.C., A.C. (Italian cohort), and E.S. (Polish cohort). M.H. (Finnish cohort); Validation, P.G.S., C.M.; Formal Analysis, M.P., A.B., M.M.V., W.S., T.G. M.E.T.D, M.H.,P.A.G., N.M., P.E.S, A.R.B; Investigation, R.G., F.P.; Resources, A.B, M.P., F.M.; Data Curation, R.G., P.G.S., F.P., D.G., L.C., M.M., M.E.T.D. M.M.V., E.J., F.N., F.D.F; M.D.R; R.Ga; A.R.B; Writing – Original Draft Preparation, R.G., Writing – Review & Editing, R.G., L.M., F.M., M.Ce., M.P., A.B., M.M.V., M.M. F.P., A.C.; Supervision, M.M., F.M.; Project Administration, A.B. F.L.;

Funding Acquisition, A.B.,R.G; All authors have read and agreed to the published version of the manuscript.

Data Availability: The datasets analyzed in this study are available from the corresponding author upon reasonable request.

Ethics Approval and Consent to Participate

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). The Ethical Committee of the IRCCS INRCA has approved the study protocol of Report-Age project, in accordance with Helsinki declaration. The trial registration number is NCT01397682. Informed consent was obtained from all subjects involved in the study.

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References

1. Mattiuzzi C, Lippi G. Worldwide disease epidemiology in the older persons. *Eur Geriatr Med*. 2020;11(1):147-153. doi:10.1007/S41999-019-00265-2.
2. Reynolds HR, Shaw LJ, Min JK, et al. Outcomes in the ISCHEMIA Trial Based on Coronary Artery Disease and Ischemia Severity. *Circulation*. 2021;144(13):1024-1038. doi:10.1161/CIRCULATIONAHA.120.049755.
3. Salam RA El, Nabil B, Saber M, Abdelwahab HA, Saber T. Prevalence of Hepatitis C Virus Seropositivity and Its Impact on Coronary Artery Disease among Egyptian Patients Referred for Coronary Angiography. *Cardiol Res Pract*. 2016;2016. doi:10.1155/2016/1623197.
4. Ntsekhe M, Baker J V. Cardiovascular Disease Among Persons Living With HIV: New Insights Into Pathogenesis and Clinical Manifestations in a Global Context. *Circulation*. 2023;147(1):83-100. doi:10.1161/CIRCULATIONAHA.122.057443.
5. Di Benedetto S, Derhovanessian E, Steinhagen-Thiessen E, Goldeck D, Müller L, Pawelec G. Impact of age, sex and CMV-infection on peripheral T cell phenotypes: results from the Berlin BASE-II Study. *Biogerontology*. 2015;16(5):631-643. doi:10.1007/s10522-015-9563-2.
6. Nikitskaya E, Lebedeva A, Ivanova O, et al. Cytomegalovirus-Productive Infection Is Associated With Acute Coronary Syndrome. *J Am Heart Assoc*. 2016;5(8). doi:10.1161/JAHA.116.003759.
7. Marcozzi S, Beltrami AP, Malavolta M. Molecular Mechanisms to Target Cellular Senescence in Aging and Disease. *Cells*. 2022;11(23). doi:10.3390/CELLS11233732.
8. Ali I, Zhang H, Zaidi SAA, Zhou G. Understanding the intricacies of cellular senescence in atherosclerosis: Mechanisms and therapeutic implications. *Ageing Res Rev*. 2024;96. doi:10.1016/J.ARR.2024.102273.
9. Vecoli C, Basta G, Borghini A, et al. Advanced glycation end products, leukocyte telomere length, and mitochondrial DNA copy number in patients with coronary artery disease and alterations of glucose homeostasis: From the GENOCOR study. *Nutr Metab Cardiovasc Dis*. 2022;32(5):1236-1244. doi:10.1016/J.NUMECD.2022.01.021.
10. Rafiq M, Liaquat A, Javed A, et al. Association of leukocyte telomere attrition in coronary artery disease in Pakistani population: A case-control study with meta-analysis. *Clin Chim Acta*. 2023;547. doi:10.1016/J.CCA.2023.117416.
11. Wang S, Zhou X, He X, et al. Suppressive effects of pterostilbene on human cytomegalovirus (HCMV) infection and HCMV-induced cellular senescence. *Virol J*. 2022;19(1). doi:10.1186/S12985-022-01954-4.
12. Wolf J, Weinberger B, Grubeck-Loebenstien B. The immunoregulatory effects of CMV-infection in human fibroblasts and the impact on cellular senescence. *Immun Ageing*. 2012;9. doi:10.1186/1742-4933-9-1.

13. Alcaraz MJ, Alcaraz A, Teruel-Montoya R, et al. Subclinical atherosclerosis and immune activation in young HIV-infected patients with telomere shortening. *Aging*. 2021;13(14):18094-18105. doi:10.18632/AGING.203350.
14. Giacconi R, Maggi F, Macera L, et al. Prevalence and loads of torquetenovirus in the European mark-age study population. *Journals of Gerontology - Series A Biological Sciences and Medical Sciences*. 2020;75(10):1838-1845. doi:10.1093/gerona/glz293.
15. Giacconi R, Maggi F, Macera L, et al. Torquetenovirus (TTV) load is associated with mortality in Italian elderly subjects. *Exp Gerontol*. 2018;112:103-111. doi:10.1016/j.exger.2018.09.003.
16. Focosi D, Spezia PG, Macera L, et al. Assessment of prevalence and load of torquetenovirus viraemia in a large cohort of healthy blood donors. *Clinical Microbiology and Infection*. 2020;26(10):1406-1410. doi:10.1016/j.cmi.2020.01.011.
17. Gore EJ, Gard L, Niesters HGM, Van Leer Buter CC. Understanding torquetenovirus (TTV) as an immune marker. *Front Med (Lausanne)*. 2023;10. doi:10.3389/FMED.2023.1168400.
18. Frye BC, Bierbaum S, Falcone V, et al. Kinetics of Torque Teno Virus-DNA Plasma Load Predict Rejection in Lung Transplant Recipients. *Transplantation*. 2019;103(4):815-822. doi:10.1097/TP.0000000000002436.
19. Giacconi R, Laffon B, Costa S, et al. Association of Torquetenovirus Viremia with Physical Frailty and Cognitive Impairment in Three Independent European Cohorts. *Gerontology*. 2023;69(6):684-693. doi:10.1159/000528169.
20. Correia LCL, Andrade BB, Borges VM, et al. Prognostic value of cytokines and chemokines in addition to the GRACE Score in non-ST-elevation acute coronary syndromes. *Clin Chim Acta*. 2010;411(7-8):540-545. doi:10.1016/J.CCA.2010.01.011.
21. Ozeren A, Aydin M, Tokac M, et al. Levels of serum IL-1 β , IL-2, IL-8 and tumor necrosis factor- α in patients with unstable angina pectoris. *Mediators Inflamm*. 2003;12(6):361-365. doi:10.1080/09629350310001633360.
22. Balbay Y, Tikiz H, Baptiste RJ, Ayaz S, Şaşmaz H, Korkmaz Ş. Circulating interleukin-1 beta, interleukin-6, tumor necrosis factor-alpha, and soluble ICAM-1 in patients with chronic stable angina and myocardial infarction. *Angiology*. 2001;52(2):109-114. doi:10.1177/000331970105200204.
23. Liu S, Wang C, Guo J, et al. Serum Cytokines Predict the Severity of Coronary Artery Disease Without Acute Myocardial Infarction. *Front Cardiovasc Med*. 2022;9:896810. doi:10.3389/fcvm.2022.896810.
24. Priyanka S, Kaarthikeyan G, Nadathur J, Mohanraj A, Kavarthapu A. Detection of cytomegalovirus, Epstein-Barr virus, and Torque Teno virus in subgingival and atheromatous plaques of cardiac patients with chronic periodontitis. *J Indian Soc Periodontol*. 2017;21(6):456. doi:10.4103/JISP.JISP_205_17.
25. Lu AT, Seeboth A, Tsai P-C, et al. *DNA Methylation-Based Estimator of Telomere Length*. Vol 11. www.aging-us.comwww.aging-us.com.

26. Bürkle A, Moreno-Villanueva M, Bernhard J, et al. MARK-AGE biomarkers of ageing. *Mech Ageing Dev.* 2015;151:2-12. doi:10.1016/J.MAD.2015.03.006.
27. Moreno-Villanueva M, Kötter T, Sindlinger T, et al. The MARK-AGE phenotypic database: Structure and strategy. *Mech Ageing Dev.* 2015;151:26-30. doi:10.1016/J.MAD.2015.03.005.
28. Moreno-Villanueva M, Capri M, Breusing N, et al. MARK-AGE standard operating procedures (SOPs): A successful effort. *Mech Ageing Dev.* 2015;151:18-25. doi:10.1016/J.MAD.2015.03.007.
29. Bustacchini S, Abbatecola AM, Bonfigli AR, et al. The Report-AGE project: a permanent epidemiological observatory to identify clinical and biological markers of health outcomes in elderly hospitalized patients in Italy. *Ageing Clin Exp Res.* 2015;27(6):893-901. doi:10.1007/s40520-015-0350-3.
30. Di Rosa M, Sabbatinelli J, Soraci L, et al. Neutrophil-to-lymphocyte ratio (NLR) predicts mortality in hospitalized geriatric patients independent of the admission diagnosis: a multicenter prospective cohort study. *J Transl Med.* 2023;21(1). doi:10.1186/S12967-023-04717-Z.
31. Corsonello A, Soraci L, Di Rosa M, et al. Prognostic Interplay of Functional Status and Multimorbidity Among Older Patients Discharged From Hospital. *J Am Med Dir Assoc.* 2022;23(3):499-506.e1. doi:10.1016/J.JAMDA.2021.07.012.
32. Di Rosa M, D'alia S, Guarasci F, et al. Cognitive Impairment, Chronic Kidney Disease, and 1-Year Mortality in Older Patients Discharged from Acute Care Hospital. *J Clin Med.* 2020;9(7):1-12. doi:10.3390/JCM9072202.
33. Maggi F, Andreoli E, Lanini L, et al. Relationships between total plasma load of torquetenovirus (TTV) and TTV genogroups carried. *J Clin Microbiol.* 2005;43(9):4807-4810. doi:10.1128/JCM.43.9.4807-4810.2005.
34. Menditto A, Protic O, Di Rosa M, Bonfigli AR, Lattanzio F, Antonicelli R. Admission high-sensitivity cardiac troponin levels as a prognostic indicator for in-hospital mortality rates in the elderly and very elderly COVID-19 patients. *Arch Gerontol Geriatr.* 2023;104:104822. doi:10.1016/J.ARCHGER.2022.104822.
35. Giacconi R, Costarelli L, Piacenza F, et al. Main biomarkers associated with age-related plasma zinc decrease and copper/zinc ratio in healthy elderly from ZincAge study. *Eur J Nutr.* 2017;56(8):2457-2466. doi:10.1007/s00394-016-1281-2.
36. Piacenza F, Giacconi R, Costarelli L, et al. Age, Sex, and BMI Influence on Copper, Zinc, and Their Major Serum Carrier Proteins in a Large European Population Including Nonagenarian Offspring From MARK-AGE Study. *The Journals of Gerontology: Series A.* May 2021. doi:10.1093/gerona/qlab134.
37. Malavolta M, Giacconi R, Piacenza F, et al. Plasma copper/zinc ratio: an inflammatory/nutritional biomarker as predictor of all-cause mortality in elderly population. *Biogerontology.* 2010;11(3):309-319. doi:10.1007/S10522-009-9251-1.

38. Kunutsor SK, Voutilainen A, Kurl S, Laukkanen JA. Serum copper-to-zinc ratio is associated with heart failure and improves risk prediction in middle-aged and older Caucasian men: A prospective study. *Nutr Metab Cardiovasc Dis*. 2022;32(8):1924-1935. doi:10.1016/J.NUMECD.2022.05.005.
39. Cabral M, Kuxhaus O, Eichelmann F, et al. Trace element profile and incidence of type 2 diabetes, cardiovascular disease and colorectal cancer: results from the EPIC-Potsdam cohort study. *Eur J Nutr*. 2021;60(6):3267-3278. doi:10.1007/S00394-021-02494-3.
40. Baldelli L, Pirazzini C, Sambati L, et al. Epigenetic clocks suggest accelerated aging in patients with isolated REM Sleep Behavior Disorder. *NPJ Parkinsons Dis*. 2023;9(1). doi:10.1038/s41531-023-00492-2.
41. Roth GA, Abate D, Abate KH, et al. Global, regional, and national age-sex-specific mortality for 282 causes of death in 195 countries and territories, 1980-2017: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet*. 2018;392(10159):1736-1788. doi:10.1016/S0140-6736(18)32203-7.
42. Tan BK, Chalouni M, Ceron DS, et al. Atherosclerotic Cardiovascular Events in Patients Infected With Human Immunodeficiency Virus and Hepatitis C Virus. *Clin Infect Dis*. 2021;72(9):E215-E223. doi:10.1093/CID/CIAA1014.
43. Mir R, Jha CK, Khullar N, et al. The Role of Inflammatory and Cytokine Biomarkers in the Pathogenesis of Frailty Syndrome. *Endocr Metab Immune Disord Drug Targets*. 2022;22(14):1357-1366. doi:10.2174/1871530322666220304220522.
44. Kundu S, Freiberg MS, Tracy RP, et al. Circulating T Cells and Cardiovascular Risk in People With and Without HIV Infection. *J Am Coll Cardiol*. 2022;80(17):1633-1644. doi:10.1016/J.JACC.2022.08.756.
45. Spyridopoulos I, Martin-Ruiz C, Hilkens C, et al. CMV seropositivity and T-cell senescence predict increased cardiovascular mortality in octogenarians: Results from the Newcastle 85+ study. *Aging Cell*. 2016;15(2):389-392. doi:10.1111/ace1.12430.
46. Dadashi M, Hajikhani B, Ghazi M, et al. The global prevalence of Chlamydia pneumoniae, Helicobacter pylori, Cytomegalovirus and Herpes simplex virus in patients with coronary artery disease: A systematic review and meta-analysis. *Microb Pathog*. 2021;152. doi:10.1016/J.MICPATH.2020.104572.
47. Kong C, Liu G, Kalady MF, Jin T, Ma Y. Dysbiosis of the stool DNA and RNA virome in Crohn's disease. *J Med Virol*. 2023;95(2). doi:10.1002/jmv.28573.
48. Xie Y, Xue Q, Jiao W, et al. Associations Between Sputum Torque Teno Virus Load and Lung Function and Disease Severity in Patients With Chronic Obstructive Pulmonary Disease. *Front Med (Lausanne)*. 2021;8. doi:10.3389/FMED.2021.618757.
49. Giacconi R, Chiodi L, Boccoli G, et al. Reduced levels of plasma selenium are associated with increased inflammation and cardiovascular disease in an Italian elderly population. *Exp Gerontol*. 2021;145. doi:10.1016/J.EXGER.2020.111219.

50. Rocchi J, Ricci V, Albani M, et al. Torquetenovirus DNA drives proinflammatory cytokines production and secretion by immune cells via toll-like receptor 9. *Virology*. 2009;394(2):235-242. doi:10.1016/j.virol.2009.08.036.
51. Al Shahi H, Shimada K, Miyauchi K, et al. Elevated Circulating Levels of Inflammatory Markers in Patients with Acute Coronary Syndrome. *Int J Vasc Med*. 2015;2015. doi:10.1155/2015/805375.
52. Fisman EZ, Benderly M, Esper RJ, et al. Interleukin-6 and the risk of future cardiovascular events in patients with angina pectoris and/or healed myocardial infarction. *Am J Cardiol*. 2006;98(1):14-18. doi:10.1016/J.AMJCARD.2006.01.045.
53. Mallat Z, Besnard S, Duriez M, et al. Protective role of interleukin-10 in atherosclerosis. *Circ Res*. 1999;85(8). doi:10.1161/01.RES.85.8.E17.
54. Liu C, Yu Z, Chen H, et al. Relationship Between Immunoinflammation and Coronary Physiology Evaluated by Quantitative Flow Ratio in Patients With Coronary Artery Disease. *Front Cardiovasc Med*. 2021;8. doi:10.3389/FCVM.2021.714276.
55. Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol*. 2004;75(2):163-189. doi:10.1189/JLB.0603252.
56. Wang H, Zhang N, Li G, Xu B. Proinflammatory cytokine IFN- γ , lncRNA BANCR and the occurrence of coronary artery disease. *Life Sci*. 2019;231. doi:10.1016/J.LFS.2019.05.066.
57. Zanotta N, Maximova N, Campisciano G, et al. Up-regulation of the monocyte chemotactic protein-3 in sera from bone marrow transplanted children with torquetenovirus infection. *J Clin Virol*. 2015;63:6-11. doi:10.1016/J.JCV.2014.11.028.
58. Noppert GA, Feinstein L, Dowd JB, et al. Pathogen burden and leukocyte telomere length in the United States. *Immun Ageing*. 2020;17(1). doi:10.1186/S12979-020-00206-9.
59. Ngwa NE, Peer N, Matsha TE, et al. Associations of leucocyte telomere length with cardio-metabolic risk profile in a South African HIV-infected population. *Medicine*. 2022;101(5):E28642. doi:10.1097/MD.00000000000028642.

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Table 1 Clinical characteristics of IHD patients and NIHD in the MARK-AGE study.

	NIHD N=900	IHD patients N=86	P value
Age (yrs)	64.3 ± 6.7	65.5± 6.8	NS
Males (%)	504 (56.6%)	61 (70.9%)	0.010
BMI (kg/m²)	26.8 ± 0.1	28.0± 0.5	0.005
Diabetes n (%)	61 (6.7%)	15 (17.4%)	0.001
Hypertension n (%)	286 (31.7%)	47 (54.6%)	<0.001
AF n (%)	70 (7.7%)	20 (23.2%)	<0.001
Previous MI (%)	0 (0%)	37 (43.0%)	<0.001
CRP (µg/L)	2.1 ± 0.1	2.8 ± 0.2	0.035
NLR	1.89 ± 0.03	1.90 ± 0.10	NS
Neutrophils (x103/µL)	3.4± 0.09	4.0± 0.10	NS
Lymphocytes (x103/µL)	1.99±0.07	2.38±0.22	NS
Cu/Zn ratio	1.57 ± 0.01	1.69 ± 0.03	0.001
Total cholesterol (mmol/L)	5.68 ± 0.03	5.13 ± 0.11	<0.001

LDL cholesterol (mmol/L)	3.42 ± 0.03	2.96 ± 0.10	<0.001
Triglycerides (mmol/L)	1.29 ± 0.03	1.40 ± 0.08	NS
CMV-IgG	40.8 ± 1.5	54.7 ± 4.8	0.006

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

The laboratory parameter analysis was adjusted for age, sex, countries

AF atrial fibrillation; BMI Body Mass Index; CRP C-reactive protein, Cu/Zn: Copper/Zinc ratio; MI myocardial infarction; NLR Neutrophil-to-lymphocyte ratio; NS Not significant

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Table 2. Multinomial Logistic Regression Analysis of Ischemic Heart Disease (IHD) in the MARK-AGE Population

Variables	Categories	IHD	P value
		OR (95% CI)	
Age		1.0 (0.97-1.05)	NS
Sex	males	3.45 (1.81-6.59)	<0.0001
	females	1 (reference)	
Countries		0.91 (0.80-1.03)	NS
BMI		1.01 (0.96-1.08)	NS
Cu/Zn		3.71 (1.63-8.42)	0.002
CMV-IgG		1.00 (0.99-1.01)	NS
CRP		1.00 (0.94-1.07)	NS
TTV viremia	<4log	1 (reference)	
	≥4log	2.51 (1.42-4.43)	0.002
Diabetes	yes	2.54 (1.22-5.26)	0.012
	no	1 (reference)	
Hypertension	yes	2.34 (1.37-3.99)	0.002
	no	1 (reference)	
Hypercholesterolemia	yes	0.68 (0.39-1.16)	NS

	no	1 (reference)	
Smoking	current	2.08 (1.13-3.84)	0.019
	no	1 (reference)	

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

BMI Body Mass Index; CMV-IgG Cytomegalovirus CRP C-reactive protein; Cu/Zn plasma

Copper/Zinc ratio; IgG Immunoglobulin G; NS Not significant

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Table 3 Multinomial Logistic Regression Analysis of Ischemic Heart Disease (IHD) in the Report-Age cohort

Variables	Categories	IHD	P value
		OR (95% CI)	
Age		1.03 (0.98-1.08)	NS
Sex	males	1.12 (0.59-2.12)	NS
	females	1 (reference)	
TTV viremia	<4log	1 (reference)	
	≥4log	4.90 (2.32-10.39)	<0.0001
CCI		1.14 (0.73-1.76)	NS
CHF		1.27 (0.55-2.95)	NS
Number of medications at discharge		1.13 (1.12-1.43)	<0.0001

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

CCI Charlson Comorbidity Index; CHF chronic heart failure; NS Not significant

Figure 1. Plasma cytokine concentrations in Ischemic heart disease (IHD) patients and non-ischemic heart disease (NIHD) subjects according to TTV viremia in the MARK-AGE study.

IFN- γ , IL-1 β , IL-6, IL-10, IL-12p70 and TNF- α increased in IHD patients and NIHD with TTV viremia $\geq 4\log$ compared to those with TTV viremia $< 4\log$

IHD with TTV viremia $\geq 4\log$ exhibited heightened cytokine production compared to NIHD with TTV viremia $< 4\log$, but this effect was not observed in comparison to NIHD with TTV viremia $> 4\log$.

ANCOVA analysis correcting for age, sex and country * $p < 0.05$; ** $p < 0.01$

Figure 2. NLR and SIRI index in the Report-Age and MARK-AGE cohorts according to TTV viremia and correlation between DNAm-LTL, age and TTV DNA loads in Report-Age participants

IHD Report-Age patients with TTV viremia $\geq 4\log$ showed significantly higher NLR and SIRI index compared to those with TTV viremia $< 4\log$ in (A, B).

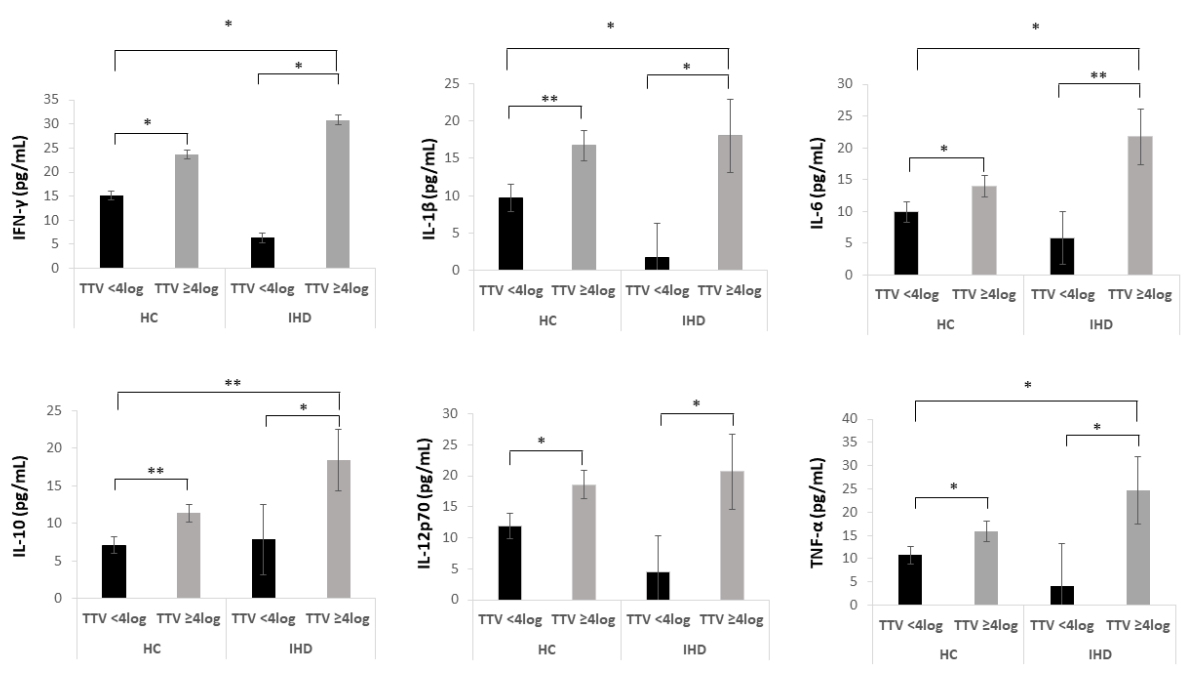
No significant differences were found in the NLR and SIRI index among subjects from the MARK-AGE non-ischemic heart disease (NIHD) group or those with ischemic heart disease (IHD), based on TTV viremia (C, D). ANCOVA analysis correcting for age and sex (A,B) or age, sex and countries (C,D) was applied; data are reported mean \pm Standard Error of the Mean (SEM) * $p < 0.05$ compared to IHD patients with TTV viremia $\geq 4\log$

(E, F) Scatter plots and Spearman correlation between DNAm-LTL, age and TTV DNA loads in Report-Age cohort

A negative correlation between DNAm-LTL and age was observed ($r=-0.366$ $P<0.001$) in Report-Age cohort (E). TTV DNA loads (log-transformed data) were negatively correlated with DNAm-LTL in both NIHD ($r=-0.172$, $p<0.05$) and IHD subjects ($r=-0.228$, $p<0.05$) (F). DNAmTL values were expressed in kilobases (Kb) and viral loads were reported as number of viral genomic copies per mL of whole blood.

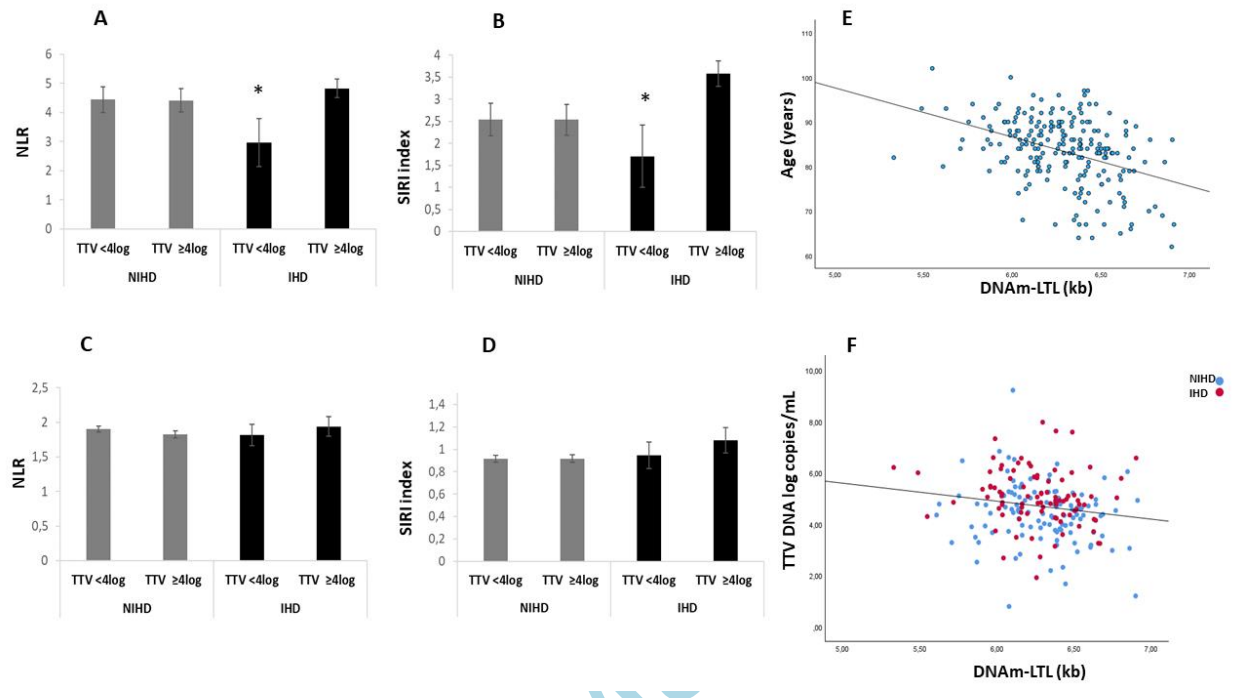
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Figure 1



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Figure 2



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