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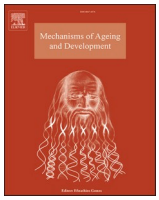

# Association of cytomegalovirus serostatus with ELOVL2 methylation: Implications for lipid metabolism, inflammation, DNA damage, and repair capacity in the MARK-AGE study population

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ABSTRACT

Cytomegalovirus (CMV) infection has been linked to accelerated biological aging, potentially increasing the risk of cardiovascular disease. DNA methylation of the gene Elongation Of Very Long Chain Fatty Acids-Like 2 (*ELOVL2*) is a molecular biomarker for aging, and its gene product is involved in polyunsaturated fatty acid synthesis, which impacts immune and inflammatory responses. This study, conducted in the MARK-AGE population, aimed to investigate the relationship between CMV infection and ELOVL2 methylation in adults aged 35–75, as well as the influence of CMV IgG levels on lipid metabolism, inflammation, DNA damage, and DNA repair. Our data revealed a higher prevalence of ischemic heart disease, atrial fibrillation, hypertension, and

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diabetes in CMV-positive individuals. CMV IgG levels were positively associated with *ELOVL2* methylation at specific CpG sites and with increased expression of DNA methyltransferase-1 (DNMT1). CMV IgG was linked to lipid imbalances, such as increased BMI, VLDL-cholesterol, triglycerides, and HDL1-cholesterol. Additionally, *ELOVL2* methylation was associated with systemic inflammation markers, lipid parameters and altered T-cell subsets. A negative correlation was observed between CMV IgG levels and both baseline DNA integrity and repair capacity. These results suggest that CMV infection might promote cardiovascular disease through *ELOVL2* hypermethylation, lipid dysregulation, inflammation, and DNA damage.

## 1. Introduction

Cytomegalovirus (CMV), a member of the beta-herpesvirus family, is a highly prevalent human pathogen that infects 50–90 % of adults in developing countries (Fowler et al., 2022). Typically acquired early in life, CMV establishes lifelong latency, with most infections in immunocompetent individuals remaining asymptomatic (Müller and Di Benedetto, 2024). However, accumulating evidence suggests that CMV infection has profound immunological and systemic effects, particularly in aging and disease progression.

One of the most well-documented consequences of CMV infection is its impact on the immune system. CMV-specific T cells accumulate over time, leading to oligoclonal expansion of $CD8^{+}CD28^{-}CD57^{+}$ T cells, which alters T-cell repertoire diversity and impairs the function of other T-cell populations. This decline in immune diversity contributes to immunosenescence, weakening the host's ability to respond to infections and potentially influencing overall health in aging individuals (Hassouneh et al., 2021; Müller and Di Benedetto, 2024). Beyond its role in immunosenescence, CMV has been implicated in the development and progression of cardiovascular diseases (CVD). It promotes a proinflammatory environment, induces oxidative stress, and may accelerate biological aging (Müller and Di Benedetto, 2024; Poloni et al., 2022). Chronic viral infections have been shown to accelerate biological aging through epigenetic changes. For example, human immunodeficiency virus (HIV) infection is associated with an average epigenetic age acceleration of 5.2 years, alongside an increased risk of CVD (Ebner et al., 2021; Horvath and Levine, 2015). The impact of cytomegalovirus (CMV) infection on epigenetic aging was first described by Kananen and coauthors (Kananen et al., 2015), who found accelerated aging in CMV-positive young individuals aged 20–30 years, as well as in the elderly aged 90 years and older. These findings have since been corroborated by other studies (Bacalini et al., 2017; Poloni et al., 2022).

One of the most robust molecular biomarkers for biological age is the methylation of the Elongation Of Very Long Chain Fatty Acids-Like 2 (*ELOVL2*) gene promoter, which increases linearly with age (Bacalini et al., 2017; Garagnani et al., 2012). The *ELOVL2* gene encodes a transmembrane protein involved in the elongation of long-chain fatty acids and the synthesis of polyunsaturated fatty acids (PUFAs), which play key roles in immune and inflammatory responses (Jakobsson et al., 2006). Dysregulation of ELOVL2 expression has been linked to metabolic and age-related diseases. Knockout studies in mice have shown that loss of ELOVL2 leads to male infertility, altered PUFA levels, insulin resistance, and disrupted lipid metabolism (Cruciani-Guglielmacci et al., 2017; Pauter et al., 2014; Zadravec et al., 2011). Moreover, increased ELOVL2 methylation with age may contribute to dyslipidemia, diabetes, and other cardiovascular risk factors (Cruciani-Guglielmacci et al., 2017; Li et al., 2022).

Decreased ELOVL2 expression is associated with impaired PUFA synthesis and the accumulation of shorter fatty acid precursors in the endoplasmic reticulum (ER), leading to mitochondrial dysfunction and chronic ER stress (Li et al., 2022). These metabolic disturbances are known to contribute to CVD pathogenesis (Navas-Madroñal et al., 2023). Additionally, PUFA synthesis, regulated by ELOVL2, plays a key role in modulating immune responses and inflammation, two critical processes in both aging and cardiovascular health (Buckley et al., 2014; Oishi et al., 2017).

Based on these observations, the aim of this study is to investigate the relationship between CMV infection and *ELOVL2* methylation in community-dwelling adults aged 35–75 years enrolled in the MARK-AGE project. Furthermore, we will explore the impact of ELOVL2 methylation on lipid metabolism, inflammation, and DNA damage and repair, providing new insights into the molecular mechanisms linking CMV infection, epigenetic aging, and cardiovascular health.

## 2. Materials and methods

### 2.1. Study population, recruitment, data and blood collection

This cross-sectional study included 1992 RASIG (Randomly recruited Age-Stratified Individuals from the General population) participants within the age bracket of 35–75 years. These subjects were recruited as part of the European MARK-AGE project (Bürkle et al., 2015; Moreno-Villanueva et al., 2015a).

The details of the recruitment procedures, along with the collection of anthropometric, clinical, demographic, and laboratory parameter data, have already been reported (Jansen et al., 2015; Moreno-Villanueva et al., 2015b, 2015a). Health status and medical history were evaluated on the basis of detailed questionnaires (Moreno-Villanueva et al., 2015a). Plasma isolation procedure from blood, as well as shipment and distribution of biological samples have been described (Moreno-Villanueva et al., 2015a). 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.

### 2.2. CMV IgG antibody titer

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

### 2.3. Measurement of ELOVL2 Methylation

Total DNA was extracted from PBMCs using the QIAamp 96 DNA Blood Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. 1 μg of DNA was subjected to bisulfite conversion using the EZ-96 DNA Methylation Kit (Zymo Research, Irvine, USA), according to the manufacturer's protocol that includes incubation in CT buffer for 21 cycles of 15 min at 55 °C and 30 s at 95 °C, followed by elution of the bisulfite-treated DNA in 100 μl of water. Bisulfite conversion transformed unmethylated cytosines into uracils, while methylated cytosines remained unchanged. PCR with bisulphite-specific primers was performed on 10 ng of converted DNA. The amplified regions were chr6:11,044,680–11,045,053 (GRCh37/hg19) within the *ELOVL2* locus. *ELOVL2* amplicons included probes: cg16867657, cg24724428, cg21572722, cg16323298 from the Infinium 450k design. PCR products were processed using the EpiTYPER assay (Sequenom) following manufacturer's instructions to measure the methylation levels at individual

CpG sites or grouped adjacent CpG units, depending on the genomic sequence (Supplemetary Figure1S) (Garagnani et al., 2012). In particular the assay provides methylation levels for the following CpG sites/units: CpG_2.3, CpG_5, CpG_6.7, CpG_8, CpG_9, CpG_10, CpG_11.12.13.14; CpG_15.16.17, CpG_18.19.20.21 CpG_22.23.24 CpG_27, CpG_28.29, CpG_30.31.32, CpG_33, CpG_34.35.36. All these CpG sites/units had less than 20 % missing values and were included in the analysis. It has been also calculated the mean methylation value of *ELOVL2* CpG sites assessed (from CpG_2.3 to CpG_34.35.36) (Bacalini et al., 2017).

### 2.4. Lipid profile analysis

The cholesterol and triglyceride content in serum lipoprotein fractions (HDL, LDL, VLDL) and subfractions (HDL2, LDL2, VLDL2) were measured using nuclear magnetic resonance (NMR) spectroscopy (Bruker Biospin), as previously described (Heijmans et al., 2006; Vaarhorst et al., 2011). Each measurement generates signal amplitudes from lipoprotein subclasses, enabling the estimation of both total lipoprotein particle concentration and subclass distribution, including small particles (HDL2, LDL2, VLDL2) (Giacconi et al., 2019).

### 2.5. Cell phenotyping

Lymphocyte subsets in PBMC samples from MARK-AGE biobank were analyzed by FACS as previously described (Dudkowska et al., 2017). 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 analyzed on the FACS Calibur (Beckton-Dickinson, Warsaw, Poland) using the Cell-Quest software (BD) and Multiset software (Beckton-Dickinson), respectively.

### 2.6. Cytokine determination

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-1β, IL-6, IL-10, IL-12p70, TNF-α, and IFN-γ).

### 2.7. Quantification of DNA strand breaks and DNA repair

We used the automated FADU assay (Fieres et al., 2022; Morath et al., 2014; Moreno-Villanueva et al., 2009) a very sensitive in vitro method, to assess DNA strand breaks and DNA repair. The detection of DNA strand breaks and repair is based on progressive DNA unwinding (denaturation) under highly controlled conditions of alkaline pH, time, and temperature. The starting points for the unwinding process are DNA strand interruptions, such as replication forks or chromosome ends, but also DNA strand breaks induced by reactive oxygen species (ROS), irradiation, or chemical compounds. For monitoring alkaline DNA unwinding, a commercially available fluorescent dye (Sybr®Green, Invitrogen, Darmstadt, Germany) is used as a marker for double stranded DNA remaining after termination of the alkaline conditions. Fluorescence intensity is expressed as a percentage of fluorescence in control cells without induced DNA damage (P0). A decrease in the fluorescence intensity of Sybr®Green indicates an increase of DNA unwinding and, consequently, a higher number of DNA strand breaks. Human peripheral blood mononuclear cells (PBMCs) were isolated from whole blood according to the density gradient principle using Biocoll® (Biochrom AG, Berlin, Germany), counted using a cell counting device (Casy® counter), pelleted (5 min, 200 *g*), and resuspended in RPMI-1640 medium (Invitrogen) containing 100 U/ml penicillin (Invitrogen) and 100 mg/ml streptomycin (Invitrogen) at $5 \times 10^5$ cells/ml. Then several 100-µl aliquots of cell suspension were irradiated on ice with 3.8 Gy (dose rate 1.9 Gy/min) using an X-ray generator (C.H.F. Müller, Hamburg, Germany, 70 keV, 1-mm Al filter). To allow DNA repair, cells were incubated in a $CO_2$ incubator at 37 °C for 40 min and subsequently transferred to the pipetting robot for the FADU assay. DNA repair was analyzed by the FADU assay.

### 2.8. Statistical analysis

Subject characteristics were reported as mean ± standard error of the mean (SEM) or percentages for continuous and categorical variables, respectively. For continuous variables, 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 One-way Analysis of Variance for continuous variables and Pearson's $\chi^2$ test for categorical variables. We performed stepwise linear regression analyses to examine the associations between ELOVL2 CpGs (from CpG_2.3 to CpG_34.35.36) and CMV IgG antibody levels, adjusting for age, gender, and country. Linear regression models were also performed to search for an association between CMV IgG antibody titer and *DNMT1* and *DNMT3* mRNA levels or lymphocyte subsets after adjusting for age, sex and country in the RASIG population. Multivariate stepwise linear regression analysis was performed to explore the associations between CMV IgG levels (dependent variable), lipid profile and laboratory parameters. The following variables were included in the analyses: age, sex, country, BMI, Charlson comorbidity index (CCI), total cholesterol, HDL-cholesterol, HDL1-cholesterol, HDL2-cholesterol, LDL-cholesterol, LDL1-cholesterol, LDL2-cholesterol, total triglycerides, HDL-triglycerides, HDL1-triglycerides, HDL2-triglycerides, LDL-triglycerides, LDL2-triglycerides, LDL1-triglycerides, VLDL-cholesterol, VLDL1-cholesterol, VLDL2-cholesterol, VLDL-triglycerides, VLDL1-triglycerides, VLDL2-triglycerides, leukocyte count, neutrophils, lymphocytes, monocytes, platelets, albumin, CRP, free fatty acids, uric acid, insulin, fasting glucose, HbA1c, ceruloplasmin, alpha-2 macroglobulin, creatinine, fibrinogen, adiponectin. A linear regression model, adjusted for age, sex, and country, was performed to examine the association between mean *ELOVL2* methylation and lymphocyte subsets in the RASIG population. All the analyses were performed using the SPSS/Win program (version 27.0; Spss Inc., Chicago, IL).

## 3. Results

### 3.1. Characteristics of the RASIG population divided based on CMV positivity

The characteristics of the study population are summarized in Table 1. CMV positive subjects were older than CMV negative ones and showed a lower proportion of males. CMV positivity was higher in the 55–64 and 65–74 age groups compared to the 35–44 age group (Table 1S, supplementary material) and in individuals with a BMI > 30 compared to those with a BMI < 25 (Table 1S, Table 2S supplementary material). CMV positive subjects also displayed an increased BMI and lymphocyte count. The other laboratory parameters showed no significant differences. Moreover, the prevalence of ischemic heart disease (IHD), hypertension, atrial fibrillation and diabetes was significantly higher in the CMV positive group compared to the CMV negative group ($p < 0.001$).

An adjusted logistic regression, accounting for age, sex, country, smoking, diabetes, hypertension, and dyslipidemia, confirms the association between CMV positivity and IHD (Supplementary Table 3S). Similar adjusted logistic regression models also confirm the association of CMV positivity with diabetes and hypertension (Supplementary Tables 4S and 5S). In summary, CMV positivity was associated with older age, higher BMI, and an increased prevalence of ischemic heart disease, hypertension, atrial fibrillation, and diabetes.

**Table 1**
Demographic and clinical characteristics of RASIG participants.

| | CMV negative n. 685 | CMV positive n. 1307 | P value |
|---|---|---|---|
| Age (yrs) | 53.6 ± 0.43 | 56.7 ± 0.31 | < 0.001 |
| Males n, (%) | 376 (52.7 %) | 611 (44.3 %) | < 0.001 |
| BMI (kg/m2) | 25.7 ± 0.2 | 26.5 ± 0.1 | < 0.001 |
| RBC (x10$^6$/ μL) | 4.76 ± 0.43 | 5.08 ± 0.35 | NS |
| WBC (x10$^3$/μL) | 5.96 ± 0.08 | 6.07 ± 0.07 | NS |
| Neutrophils (x10$^3$/μL) | 3.43 ± 0.06 | 3.35 ± 0.05 | NS |
| Lymphocytes (x10$^3$/μL) | 1.85 ± 0.03 | 2.02 ± 0.02 | < 0.001 |
| Monocytes (x10$^3$/μL) | 0.48 ± 0.01 | 0.49 ± 0.01 | NS |
| Platelets (x10$^3$/μL) | 236.3 ± 2.2 | 236.2 ± 1.8 | NS |
| Albumin (g/dL) | 4.07 ± 0.01 | 4.08 ± 0.01 | NS |
| CRP (μg/L) | 1.94 ± 0.11 | 2.12 ± 0.09 | NS |
| TC (mmol/L) | 5.50 ± 0.04 | 5.56 ± 0.03 | NS |
| HDL (mmol/L) | 1.51 ± 0.01 | 1.52 ± 0.01 | NS |
| LDL (mmol/L) | 3.28 ± 0.03 | 3.31 ± 0.03 | NS |
| TG (mmol/L) | 1.21 ± 0.03 | 1.29 ± 0.02 | 0.062 |
| FG (mmol/L) | 5.20 ± 0.04 | 5.22 ± 0.03 | NS |
| HbA1c (%) | 6.00 ± 0.02 | 5.96 ± 0.02 | NS |
| Creatinine (μmol/L) | 73.4 ± 0.50 | 74.0 ± 0.40 | NS |
| IHD n, (%) | 19 (2.6 %) | 79 (5.7 %) | 0.002 |
| AF n, (%) | 42 (5.9 %) | 118 (8.6 %) | 0.029 |
| Hypertension n, (%) | 114 (16.0 %) | 355 (25.7 %) | < 0.001 |
| Diabetes n, (%) | 18 (3.4 %) | 74 (5.2 %) | < 0.01 |
| Depressive symptoms n, (%) | 82 (11.7 %) | 173 (12.7 %) | NS |
| Osteoporosis n, (%) | 44 (6.2 %) | 127 (8.2 %) | NS |
| Hypothyroidism n, (%) | 58 (8.1 %) | 110 (9.2 %) | NS |

Data are reported as mean ± standard error of the mean (SEM). The laboratory parameter analysis was adjusted for age, gender, country. AF= Atrial fibrillation; BMI Body Mass Index; CRP = C-reactive protein; FG = fasting glucose; HbA1c = Hemoglobin A1c; HDL = high-density lipoprotein cholesterol; IHD= Ischemic heart disease; LDL = low-density lipoprotein cholesterol; RBC = red blood cells; TC = total cholesterol; TG = triglycerides; WBC = white blood cells.

### 3.2. Multivariate stepwise linear regression analysis for ELOVL2 CpG site methylation independently associated with CMV IgG antibody levels in the RASIG population

Linear regression analysis using the stepwise method was carried out to explore the main predictors of CMV IgG antibody levels. The variables included were: age, sex, country and all *ELOVL2* CpGs from CpG_2.3 to CpG_34.35.36. A positive association was found with *ELOVL2* CpG_18.19.20.21 and *ELOVL2* CpG_6.7, while *ELOVL2* CpG_34.35.36 was negatively associated with CMV IgG levels (Table 2). An association was also observed between CMV IgG antibody titer, country, and sex (Table 2). In particular, the lowest CMV IgG titer was observed in Germany, while Poland, Italy, and Greece showed the highest values (Supplementary table 6S). Moreover, females had higher CMV IgG levels than males (Supplementary table 6S). After sex stratification, the association between CMV IgG and both *ELOVL2* CpG_18.19.20.21 and *ELOVL2* CpG_34.35.36 was confirmed in both sexes. Additionally, a negative association between CMV IgG and *ELOVL2* CpG_30.31.32 was observed in females, while a positive association between CMV IgG and *ELOVL2* CpG_28.29 was found in males (Supplementary Table 7S). In summary, CMV IgG antibody levels were associated with specific ELOVL2 CpG sites, sex, and country. Notably, ELOVL2 CpG_18.19.20.21 and CpG_6.7 showed a positive association with CMV IgG levels, while CpG_34.35.36 was negatively associated.

**Table 2**
Multivariate stepwise linear regression analysis showing the association between ELOVL2-methylation and CMV IgG levels in the RASIG population.

| | β coefficient | 95 % C.I. | P value |
|---|---|---|---|
| ELOVL2 CpG_18.19.20.21 | 1.223 | 0.818–1.628 | < 0.0001 |
| ELOVL2 CpG_34.35.36 | −1.116 | −1.574 to −0.658 | < 0.0001 |
| ELOVL2 CpG_6.7 | 0.465 | 0.191–0.738 | 0.001 |

Adjusted for age, sex and country

### 3.3. Multivariate linear regression analysis between CMV IgG antibody levels and DNA methyltransferases in the RASIG population

A linear regression model, adjusted for age, sex, and country, identified a positive association between CMV IgG antibody levels and DNMT1 (β Coefficient = 0.336, $p < 0.001$) and a negative association with DNMT3 (β Coefficient = −0.168, $p < 0.05$) (Table 3). CMV IgG levels were associated with DNMT1 and DNMT3, suggesting a potential link between CMV infection and DNA methylation regulation.

### 3.4. Association between CMV IgG antibody levels, lipid profile and laboratory parameters

Multivariate stepwise linear regression analysis was performed to explore the associations between CMV IgG levels (dependent variable) lipid profile and laboratory parameters.

We found a positive association with VLDL-cholesterol, HDL1-cholesterol, BMI, lymphocyte count, total triglycerides, $\alpha_2$- macroglobulin, CCI. A negative association was observed between CMV IgG levels, VLDL-triglycerides, LDL2-triglycerides and HDL-cholesterol (Table 4). These findings highlight the complex relationship between CMV infection and lipid metabolism.

### 3.5. Association between ELOVL2 methylation with lymphocyte subsets, lipid parameters and inflammatory markers in RASIG population

In a subgroup of 1489 participants with a mean age of 55.1 ± 12.8 years, comprising 755 females and 734 males, we explored the association between the mean *ELOVL2* methylation of all CpG units (from CpG_2.3 to CpG_34.35.36) and lymphocyte subsets. Interestingly, a negative association with CD4 lymphocytes and a positive association with CD8 cells were observed. No association was found between *ELOVL2* methylation and NK cells or B cells (Table 5). The relationship between ELOVL2 methylation and lipid parameters was evaluated by a stepwise linear regression in the whole sample (Table 6). All ELOVL2 CpG units were associated with at least one lipid parameter. In particular, ELOVL2_CpG_15.16.17, ELOVL2_CpG_22.23.24, ELOVL2_CpG_28.29, and ELOVL2_CpG_34.35.36 showed the strongest associations, being linked to multiple lipoprotein fractions and subfractions. Regarding the relationship between *ELOVL2* methylation and inflammation in the whole population, we found a positive correlation with the CRP to albumin ratio (CAR), CRP, fibrinogen, homocysteine, and $\alpha_2$-macroglobulin (Supplementary Table 8S). In a subgroup of 207 RASIG subjects, we measured several cytokines (IL-1β, IL-6, IFN-γ) and the chemokine MCP-1, all of which showed a positive correlation with the mean *ELOVL2* methylation of all CpG units (Supplementary Figure 2S).

In summary, ELOVL2 methylation was negatively associated with CD4 lymphocytes and positively with CD8 lymphocytes, correlated with various lipid parameters and inflammation markers.

### 3.6. Association between CMV IgG levels baseline DNA integrity and DNA repair capacity in PBMCs

Baseline DNA integrity and repair were assessed in PBMCs from RASIG participants (N = 1710 subjects, 862 females and 848 males, mean age: 55.3 ± 11.4 years) using the automated FADU assay. In Fig. 1 (A) it is shown that higher CMV IgG levels were associated with reduced intact DNA, reflecting increased baseline DNA damage (Linear regression, β = −0.063; p = 0.012, adjusted for age, sex, and country). In Fig. 1 (B), DNA repair capacity following 3.5 Gy X-irradiation is reported across percentiles of CMV IgG levels. Elevated CMV IgG levels were linked to impaired DNA repair (Linear regression, β = −0.058; p = 0.020, adjusted for age, sex, and country). The negative values of

**Table 3**
Linear regression models of CMV IgG levels with DNMT1 and DNMT3 mRNA levels.

| | Unadjusted model | | Model 1 | | Model 2 | |
|---|---|---|---|---|---|---|
| | *β* Coefficient (95 % CI) | *p* Value | *β* Coefficient (95 % CI) | *p* Value | *β* Coefficient (95 % CI) | *p* Value |
| DNMT1 | 0.345 (0.176–0.514) | < 0.001 | 0.336 (0.169–0.504) | < 0.001 | 0.308 (0.144–0.472) | < 0.001 |
| DNMT3 | −0.165 (−0.304–0.026) | 0.020 | −0.168 (−0.307 to −0.03) | 0.017 | −.161 (−0.297 to −0.026) | 0.020 |

Model1: adjusted for age and sex; Model 2: adjusted for age, sex and country

**Table 4**
Multivariate stepwise linear regression analysis showing the associations between CMV IgG levels, lipid profile and laboratory parameters in the RASIG population.

| | β coefficent | 95 % C.I. | P value |
|---|---|---|---|
| VLDL-Cholesterol | 0.573 | 0.179–0.967 | < 0.01 |
| HDL1-Cholesterol | 0.949 | 0.443–1.456 | < 0.0001 |
| BMI | 0.045 | 0.024–0.066 | < 0.0001 |
| Lymphocyte count | 0.452 | 0.200–0.704 | < 0.0001 |
| VLDL-Triglycerides | −1.067 | −1.456 to −0.657 | < 0.0001 |
| Total Triglycerides | 2.040 | −1.225–2.855 | < 0.0001 |
| LDL2-Triglycerides | −0.539 | −0.800 to −0.277 | < 0.0001 |
| α−2macroglobulin | 0.469 | 0.110–0.828 | 0.010 |
| HDL-Cholesterol | −1.406 | −2.470 to −0.341 | 0.010 |
| CCI | 0.127 | 0.017–0.237 | 0.024 |

Adjusted for age, sex and country
VLDL: Very low-density lipoprotein; LDL:Low-density lipoprotein; HDL: high-density lipoprotein; CCI: Charlson Comorbidity Index

**Table 5**
Linear regression model of mean ELOVL2 methylation with lymphocyte subsets in RASIG population.

| | β coefficient | 95 % C.I. | P value |
|---|---|---|---|
| CD16 + CD56 + NK cells | −0.005 | −0.020–0.009 | NS |
| CD19 + B-cells | −0.001 | −0.013–0.012 | NS |
| CD3 + T-cells | −0.020 | −0.117–0.077 | NS |
| CD4 + T-cells | −0.075 | −0.111 to −0.038 | < 0.0001 |
| CD8 + T-cells | 0.065 | 0.044–0.086 | < 0.0001 |

Model: adjusted for age, sex and country

DNA repair are likely due to very high DNA damage after irradiation that overwhelms the repair machinery, preventing the expected increase in fluorescence as DNA is repaired. This suggests a reduced DNA repair capacity, particularly in individuals with higher CMV IgG levels. In summary, elevated CMV IgG levels were linked to increased DNA damage and a diminished ability to repair DNA, indicating a potential impact on genomic stability in these individuals.

## 4. Discussion

Recent studies indicate that chronic viral infections can accelerate epigenetic aging (Oltmanns et al., 2023; Poloni et al., 2022). The epigenetic age acceleration is linked to an increased risk of cardiovascular disease (CVD) and all-cause mortality (Ammous et al., 2021; Hillary et al., 2020; McCrory et al., 2021; Wang et al., 2021). Poloni (Poloni et al., 2022) found that CMV-positive adults have a higher average epigenetic age compared to CMV-seronegative individuals. Large population studies have also shown a significant association between CMV seropositivity and an elevated risk of CVD (Gkrania-Klotsas et al., 2012; Savva et al., 2013; Spyridopoulos et al., 2016), though there are conflicting results regarding the increased risk of CVD mortality (Chen et al., 2021; Chu et al., 2022; Spyridopoulos et al., 2016). In our study, we observed a higher prevalence of ischemic heart disease (IHD), atrial fibrillation (AF), hypertension, and diabetes among CMV-positive subjects compared to CMV-negative ones. We found a positive association between CMV IgG levels and the global *ELOVL2* methylation (mean of all CpG sites) with a more significant positive association with *ELOVL2* CpG_18.19.20.21 and *ELOVL2* CpG_6.7.

**Table 6**
Multivariate Stepwise linear regression analysis of ELOVL2 methylation and its association with plasma lipid parameters in the RASIG population.

| ELOVL2 CpG units | Lipid parameters | β coefficient | P value |
|---|---|---|---|
| **ELOVL_2_CpG_2.3** | Total Cholesterol | 0.167 | < 0.001 |
| | LDL1 Triglycerides | −0.095 | < 0.01 |
| | HDL Triglycerides | 0.062 | < 0.05 |
| **ELOVL_2_CpG_5** | LDL2 Cholesterol | 0.119 | < 0.01 |
| **ELOVL_2_CpG_6.7** | Total Triglycerides | 0.070 | < 0.01 |
| **ELOVL_2_CpG_8** | LDL1 Triglycerides | −0.138 | < 0.001 |
| | VLDL2 Cholesterol | 0.117 | < 0.001 |
| | HDL1 Cholesterol | 0.052 | < 0.01 |
| **ELOVL_2_CpG_9** | Total Triglycerides | 0.051 | < 0.01 |
| **ELOVL_2_CpG_10** | HDL Triglycerides | 0.080 | < 0.01 |
| **ELOVL_2_CpG_11.12.13.14** | HDL2 Triglycerides | 0.039 | < 0.05 |
| **ELOVL_2_CpG_15.16.17** | Total Cholesterol | 0.081 | < 0.001 |
| | LDL1 Triglycerides | −0.105 | < 0.001 |
| | HDL2 Triglycerides | 0.054 | < 0.01 |
| **ELOVL2 CpG_18.19.20.21** | VLDL1 Triglycerides | 0.143 | < 0.01 |
| | Total Triglycerides | −0.108 | < 0.05 |
| **ELOVL_2_CpG_22.23.24** | VLDL2 Cholesterol | −0.259 | < 0.001 |
| | LDL1 Triglycerides | 0.184 | < 0.001 |
| | HDL1 Triglycerides | −0.104 | < 0.001 |
| | VLDL Cholesterol | 0.244 | < 0.001 |
| | Free fatty acids | 0.044 | < 0.05 |
| **ELOVL_2_CpG_27** | LDL2 Cholesterol | 0.057 | < 0.05 |
| **ELOVL_2_CpG_28.29** | VLDL2 Cholesterol | −0.255 | < 0.001 |
| | VLDL2 Triglycerides | 0.325 | < 0.001 |
| | Total Triglycerides | −0.120 | < 0.01 |
| | Free fatty acids | 0.067 | < 0.01 |
| | HDL Cholesterol | −0.060 | < 0.05 |
| **ELOVL_2_CpG_30.31.32** | LDL2 Triglycerides | 0.069 | < 0.01 |
| **ELOVL_2_CpG_33** | VLDL1 Cholesterol | 0.099 | < 0.01 |
| **ELOVL2 CpG_34.35.36** | VLDL2 Cholesterol | −0.203 | < 0.001 |
| | VLDL2 Triglycerides | 0.245 | < 0.001 |
| | Total Cholesterol | −0.310 | < 0.001 |
| | Free fatty acids | 0.066 | < 0.01 |
| | LDL Cholesterol | 0.106 | < 0.05 |

Model: adjusted for age, sex and country

*ELOVL2*, in addition to being a biomarker for chronological age (Bacalini et al., 2017; Garagnani et al., 2012), is involved in the synthesis of PUFAs. PUFAs influence inflammation by acting as precursors of eicosanoids. Notably, eicosanoids derived from n-3 PUFAs have demonstrated anti-inflammatory effects and can lower the production of inflammatory cytokines, (Darwesh et al., 2019) modulate lipogenesis, ER stress, and mitochondrial dysfunction in chronic diseases (Okada et al., 2018). In addition, a recent meta-analysis demonstrates the beneficial effects of n-3 PUFAs on coronary heart disease (Xiao et al., 2024). Accordingly, in our cohort we found a positive association between *ELOVL2* methylation, markers of systemic inflammation (CRP/albumin ratio CRP, fibrinogen and homocystein), lipoprotein fractions and subfractions, and certain plasma cytokines and chemokines (IL-1β, IL-6, IFN-γ, MCP-1).

CMV infection remodels the host lipidome, regulating fatty acid elongation and balancing SFAs, MUFAs, and PUFAs (Xi et al., 2019), also

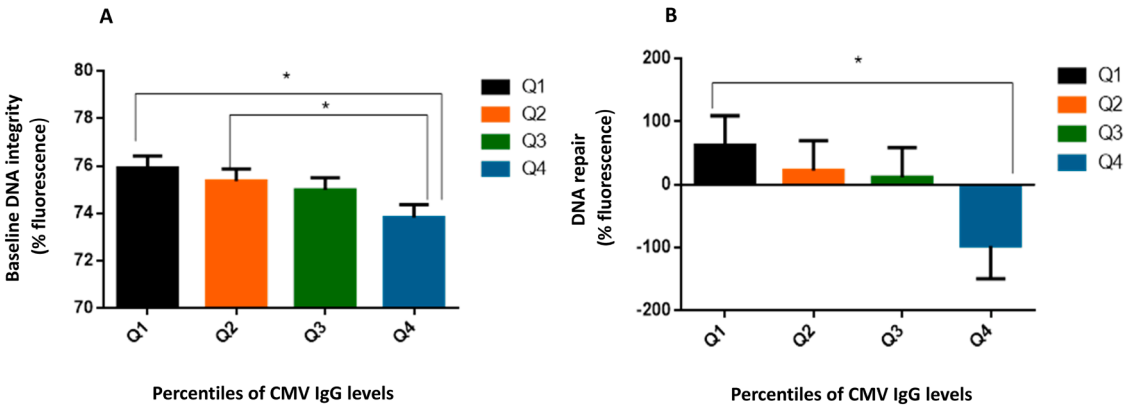


**Fig. 1.** Baseline DNA integrity, DNA repair capacity according to percentiles of CMV IgG levels Baseline DNA integrity and DNA repair was measured in PBMCs from RASIG participants using the automated FADU assay. Fig. 1 **(A)** Increased CMV IgG levels was associated with lower intact DNA and thus higher baseline DNA damage (Linear regression, $\beta = -0.063$; $p = 0.012$ adjusted for age, sex and country). **(B)** DNA repair after 3.5 Gy X-irradiation according to percentiles of CMV IgG levels.Increased CMV IgG levels showed a decreased DNA repair capacity (Linear regression, $\beta = -0.058$; $p = 0.020$, adjusted for age, sex and country).*$p < 0.05$.

inducing several lipogenic enzymes involved in fatty acid synthesis and elongation, which are crucial for CMV replication (Koyuncu et al., 2013; Purdy et al., 2015; Spencer et al., 2011).

*In vitro* experiments have further demonstrated that knockdown of *ELOVL2* inhibits virus replication (Koyuncu et al., 2013). CMV induces *ELOVL7* to synthesize the saturated VLCFA required for membrane biogenesis and efficient virus replication (Purdy et al., 2015), while there is no evidence on the effect of CMV on *ELOVL2* expression.

In our study, we have demonstrated an association between CMV IgG levels, *ELOVL2* methylation and the expression of *DNMT1* and *DNMT3* genes, with implications for lipid profile modulation and inflammation. Some evidence shows that overexpression of DNMT1, a key enzyme in DNA methylation maintenance (Howell et al., 2001), is linked to reduced PPAR-γ and increased proinflammatory cytokines in peripheral blood monocytes of atherosclerosis patients (Yu et al., 2016). Additionally, disturbed blood flow upregulates DNMT, leading to genome-wide DNA methylation changes that alter endothelial gene expression, regulate vascular biology, and promote atherosclerosis (Dunn et al., 2015).

Other findings support the idea that DNMT1 may be upregulated by viral infections (Ksiaa et al., 2014; Youngblood and Reich, 2008), though specific studies on CMV are lacking.

Consistent with other research, we observed that CMV IgG levels were positively associated with BMI, VLDL-cholesterol, and total triglycerides, known risk factors for coronary atherosclerosis, while there was a negative association with HDL-cholesterol (Di Giorgi et al., 2021; Feng et al., 2023; Pita López et al., 2023).

Interestingly, we found a positive association with the HDL1-cholesterol subfraction, which has been reported to increase with age due to reduced hepatic catabolism (Bravo et al., 1994). Moreover, VLDL- and LDL2-triglyceride subfractions showed a negative correlation with CMV IgG levels. These findings strengthen the evidence for CMV's involvement in lipid metabolism, as highlighted by several studies (Gao et al., 2023; Xin and Zhou, 2024; Xi et al., 2019).

Previous evidence suggests that *ELOVL2* deficiency accelerates aging by disrupting metabolism, leading to the accumulation of short-chain fatty acids, ER stress, mitochondrial dysfunction, and increased inflammatory mediators (Li et al., 2022), all of which can cause DNA damage (Bauer and De la Fuente, 2016; Schank et al., 2021; Xiang et al., 2024). Notably, we observed a negative association between CMV IgG levels and both the baseline DNA integrity and DNA repair capacity. This may be attributed to low ELOVL2 expression, which increases ER stress and inflammation, but could also be a direct consequence of CMV infection.

Indeed, CMV infection has been shown to induce specific types of DNA damage (Smolarz et al., 2015), and an *in vitro* study demonstrated that infected fibroblasts have a reduced ability to repair cellular DNA damage (O'Dowd et al., 2012). This could explain the observed negative association between CMV IgG levels and DNA repair capacity. Other findings in our study include a negative correlation between the mean *ELOVL2* methylation at all CpG sites and CD4 lymphocytes, and a positive correlation with CD8 cells. These results partially align with previous research showing that *ELOVL2*-deficient mice exhibit a dysregulation in cytotoxic CD8 + T cells, which produce higher levels of pro-inflammatory cytokines (Talamonti et al., 2023).

Our study has some limitations. Most notably, its cross-sectional design only allows us to identify associations between CMV infection, ELOVL2 methylation, and markers of lipid metabolism, inflammation, and DNA damage, without establishing causal relationships. We acknowledge that the absence of in vivo or in vitro data limits our ability to draw definitive conclusions regarding the mechanisms through which CMV infection may influence ELOVL2 methylation and subsequent metabolic alterations. To address these gaps, future studies with longitudinal cohorts or experimental models are needed to directly investigate the causal pathways between CMV infection, epigenetic changes, and their effects on lipid dysregulation and cardiovascular disease.

Additionally, despite adjusting for multiple confounders, unmeasured factors may still have influenced our findings.

In conclusion, elevated CMV IgG levels are associated with *ELOVL2* hypermethylation, plasma lipid alterations, increased inflammation, heightened DNA damage, and reduced DNA repair capacity, all of which may contribute to the onset and progression of cardiovascular disease. Further studies are needed to clarify the mechanisms by which CMV influences *ELOVL2* methylation and lipid metabolism.

## 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). Informed consent was obtained from all subjects involved in the study.

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## CRediT authorship contribution statement

**Bürkle Alexander:** Writing – review & editing, Project administration, Funding acquisition. **Olivieri Fabiola:** Writing – review & editing, Supervision. **Badillo Pazmay Gretta Veronica:** Writing – review & editing. **Villanueva María Moreno:** Writing – review & editing, Methodology. **Ciccarone Fabio:** Formal analysis, Data curation. **Giacconi Robertina:** Writing – review & editing, Writing – original draft, Software, Conceptualization. **Caiafa Paola:** Formal analysis. **Debacq-Chainiaux Florence:** Resources. **Bacalini Maria Giulia:** Writing – review & editing, Data curation. **Reale Anna:** Data curation. **Pirazzini Chiara:** Writing – review & editing, Data curation. **Hurme Mikko:** Resources. **Capri Miriam:** Resources, Formal analysis. **Stuetz Wolfgang:** Formal analysis. **Garagnani Paolo:** Writing – review & editing, Supervision. **Bernhardt Jürgen:** Resources. **Fortunato Carlo:** Writing – review & editing, Conceptualization. **Slagboom P. Eline:** Formal analysis. **Franceschi Claudio:** Resources, Formal analysis. **Zampieri Michele:** Formal analysis, Data curation. **Sikora Ewa:** Resources. **Weinberger Birgit:** Resources. **Piacenza Francesco:** Writing – review & editing, Data curation. **Cardelli Maurizio:** Writing – review & editing, Conceptualization. **Cianfruglia Laura:** Writing – review & editing. **Cecati Monia:** Writing – review & editing. **Jansen Eugène:** Formal analysis, Data curation. **Dollé Martijn E. T.:** Formal analysis, Data curation. **Gonos Efstathios S.:** Resources, Formal analysis. **Grune Tilman:** Resources, Formal analysis. **Malavolta Marco:** Writing – review & editing, Supervision.

## Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.mad.2025.112043.

## Data availability

Data will be made available on request.

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