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ELOVL2-methylation and renal and cardiovascular event in patients with chronic kidney disease

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Abstract

Background: Methylation of the Elongation Of Very Long Chain Fatty Acids-Like 2 (ELOVL2) gene promoter may predict premature ageing and cardiovascular risk.

Methods: We studied the cross-sectional associations between blood ELOVL2-methylation and cardiovascular risk factors in 350 patients with chronic kidney disease (CKD) stage G2–G4 aged between 22 and 90 years. In a follow-up study for a mean of 3.9 years, we investigated the association between baseline ELOVL2-methylation and renal or cardiovascular events including death.

Results: ELOVL2-methylation at seven CpG cites increased with age (the correlation coefficients between 0.67 and 0.87, $p < 0.001$). The ELOVL2-CpGs methylation was lower in patients with CKD stage G2 versus those in stage G3a, G3b and G4, but the differences were explained by age. ELOVL2-CpGs methylation showed no correlations with cardiovascular risk factors after adjusting for age. During the follow-up, 64 patients showed deterioration in renal function or died and 77 showed cardiovascular events or died. The hazard ratio and 95% confidence intervals for renal or cardiovascular events according to baseline ELOVL2-CpGs methylation were not significant after adjustment for covariates.

Conclusions: ELOVL2-hypermethylation showed a strong association with age, but was not independently associated with cardiovascular risk factors or with future renal or cardiovascular events in patients with CKD. ELOVL2 gene methylation is not likely to be itself a cause for ageing or illnesses, but it could be rather influenced by other upstream processes that deserve investigation.

KEYWORDS
ageing, cardiovascular disease, chronic kidney disease, DNA-methylation, ELOVL2, fatty acids

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1 | INTRODUCTION

Ageing is associated with extensive epigenetic remodelling. Methylation of DNA at specific loci is a promising biomarker to predict premature diseases[1] or longevity. There is currently insufficient evidence to translate epigenetic markers to disease prediction or prevention. Moreover, it is uncertain whether CpGs-methylation of specific genes may proceed the disease and affect biological processes that accelerate disease progression.

Methylation of the Elongation Of Very Long Chain Fatty Acids-Like 2 (ELOVL2) gene promoter show a linear increase with increasing age and therefore, it is one of the most robust molecular biomarkers for chronological age.[2–4] ELOVL2 catalyses the first and rate-limiting reaction of the four reactions that constitute the long-chain fatty acids elongation cycle. This process allows the addition of two carbons to the chain of long- and very long-chain fatty acids per cycle. Elovl2 protein functions as an elongase for long-chain polyunsaturated fatty acids (PUFAs), precursors of 22:6n-3, docosahexaenoic acid (DHA) and very-long chain PUFAs.[5] ELOVL2 may contribute to ageing on a cellular level by altering lipid metabolism.[6–8] Knock out of ELOVL2 gene in mice has been shown to cause infertility in male, disturbed serum levels of PUFAs,[9] insulin resistance and disturbed lipid metabolism.[8,10] Hypermethylation of ELOVL2 gene with age may cause lower expression level of the ELOVL2 mRNA and protein[4] and may thus show associations with dyslipidemia, diabetes or other cardiovascular risk factors.

A longitudinal study among 19 Dutch participants (aged 22–57 years at recruitment) has shown that ELOVL2-methylation in whole blood increased progressively over 5 years.[2] ELOVL2 hypermethylation in whole blood showed no association with longevity or mortality in the Leiden Longevity Study cohort (mean age of 94 years; range 89–104 years), suggesting no prognostic value of investigating whole blood ELOVL2-methylation.[2] In contrast, studies using telomere length or epigenetic clock according to Hannum et al.,[11] found associations between ageing markers (i.e., shorter telomere length) and poorer test results for physical and mental fitness[12] and higher mortality[13] among subjects aged 70–90 years from the Lothian Birth Cohorts. However, due to high age of the participants at recruitment, there is an inherent selection bias. Moreover, most studies did not account for conventional risk factors other than sex and age.

A key question remains whether ELOVL2-methylation as a single-locus model corresponds with the presence of risk factors, diseases and clinical outcomes. In an explorative cross sectional investigation of 350 patients suffering from chronic kidney disease (CKD) (age range 22–90 years), we studied whether ELOVL2-methylation is associated with main cardiovascular risk factors at baseline. Moreover, we investigated whether baseline ELOVL2-methylation may predict renal or cardiovascular outcomes including death during a mean follow-up period of 3.9 years.

2 | METHODS

2.1 | Subjects and design

The Cardiovascular and Renal Outcome in CKD 2–4 patients– The Fourth Homburg Evaluation (CARE FOR HOMe) study is a prospective observational study that intended to identify novel risk factors for cardiovascular events and CKD progression.[14,15] The study recruited patients with CKD stage G2–G4 according to classification of The National Kidney Foundation Kidney Disease Outcomes Quality Initiative 2002 (NKF KDOQI)™. Between 2008 and 2015, the study recruited 576 patients from the outpatient clinic at the Department of Internal Medicine IV, the division of Nephrology, Saarland University Hospital, Germany.

CKD was defined according to Kidney Disease: Improving Global Outcomes (KDIGO) guidelines and patients in stages G2 through G4 or an estimated glomerular filtration rate (eGFR) between 15 and 89 mL/min/1.73 m^2 were eligible. eGFR was estimated by Modification of Diet in Renal Disease 4 equation (MDRD). The definition of the CKD stage requires in addition to having an eGFR in the range between 60 and 90 mL/min/1.73 m^2 an additional evidence for chronic renal dysfunction. We considered the following conditions as additional evidence for CKD; the presence of proteinuria >300 mg/g; the presence of albuminuria defined as >17 mg/g (in men) and > 25 mg/g (in women); a persisted glomerular hematuria; elevated plasma cystatin C concentrations (>1.05 mg/L); elevated plasma concentrations of creatinine (>1.2 mg/dL for men and >0.9 mg/dL for women); renal disease confirmed by a biopsy; or tubulopathy.

Exclusion criteria were CKD stage G1 and G5 (eGFR >90 mL/min/1.73 m^2 and eGFR <15 mL/min/1.73 m^2, respectively), using immune suppressive drugs, acute infections [defined as C-reactive protein (CRP) above 50 mg/L and or the use of systemic antibiotics], active malignancies, acute renal dysfunction defined as an increase of plasma creatinine by >50% within 4 weeks, pregnancy and age below 18 years.[14]

The study protocol was reviewed and approved by the Ethical Committee of the Saarland (Ärztekammer des Saarlandes, approval number 08/10) and the study has been carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki)

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for medical research involving human subjects. All participants provided written informed consent to the study.

2.2 | Baseline clinical investigations

A standardised questionnaire was used to collect information on prevalent diabetes mellitus, current use of medications, a history of smoking, family history of premature cardiovascular events and existing cardiovascular comorbidities. Cardiovascular comorbidities included information on a history of myocardial infarction, coronary artery angioplasty, stenting or bypass surgery, major stroke, carotid endarterectomy or stenting, nontraumatic lower extremity amputation or lower limb artery bypass surgery, angioplasty or stenting. Diabetes was defined as self-reported diabetes mellitus, a fasting blood glucose level of at least 126 mg/dL and/or a current use of glucose-lowering drugs. Subjects were considered as active smokers if they were current smokers or had stopped smoking less than 1 month before entry into the study. People were considered non-smokers if they never smoked or had stopped smoking longer than 1 month before recruitment into the study. Blood pressure was measured during the baseline visit at the study center after at least 15 min resting time. Body weight and height were measured in all patients at the study center during the baseline visit. Body mass index (BMI) was calculated as [weight (kg)/[height (m)^2].

2.3 | Biomarkers measured at baseline visit

Blood samples were collected during the baseline visit after an overnight fasting and after 5 min of resting. Blood was collected into EDTA- and heparin-containing tubes for plasma and into dry tubes for serum. Blood samples were immediately sent to the central hospital lab for centrifugation and measurement of cystatin C, troponin T, amino-terminal pro-brain natriuretic peptide (NT-proBNP), creatinine (traceable to IDMS), glucose, lipids, CRP and a complete blood count. These markers were measured in heparin plasma samples using routine methods at the Central Laboratory of the University Hospital of the Saarland. EDTA whole blood samples were stored at −80°C for DNA isolation.

2.4 | Clinical outcomes (follow-up)

The CARE FOR HOMe study planned to follow-up the patients for duration of 5 years with yearly on-site clinical and laboratory investigation to evaluate clinical and immunological progression factors of the CKD. For patients who were unable to attend one or more of the follow-up visits, the participants were asked to fill a study-questionnaire during a follow-up phone interview where they had to answer questions about their health condition. In addition, medical investigations, reports and results of laboratory investigations were obtained from the family physicians. In patients whose renal function deteriorated and they became on dialysis, we contacted both the patients and their nephrologists to obtain the medical reports.

The primary outcomes of the CARE FOR HOMe study were, (1) cardiovascular event and all-cause death combined; (2) all cause death and worsening of renal function [defined as lowered eGFR by 50% or transfer to end stage renal disease (ESRD)]. The secondary outcomes were; all cause death; cardiovascular events and cardiovascular death; and heart failure combined with all-cause death.

Of the 575 patients who were recruited into the study, EDTA-blood samples for DNA isolation were not collected from the first 225 patients. Therefore, DNA samples were available from 350 participants at baseline visit. Those patients constituted the participants of the present study. The mean (SD) of follow-up time in the present study was 3.9 (1.8) years.

2.5 | Methods of measuring ELOVL2 gene methylation

DNA was extracted from EDTA whole blood using the QIAmp DNA blood Mini Kit (QIAGEN, Hilden, Germany). Bisulfite conversion of DNA was performed on 20 µL of 0.5 µg/mL genomic DNA, using the EZ DNA-Methylation-Lightning™ Kit (ZYMO RESEARCH, Cat Nr. 5030) according to the manufacturer instructions (Table S1). DNA bisulfite converted the non-methylated cytosine in the native DNA to uracil. The DNA-bisulfit product was diluted to a final concentration of 20 ng/µL for methylation analysis by pyrosequencing. The primer pairs used for PCR on bisulfite-treated DNA were the same described by Zbiec-Piekarska et al.,[16] (Invitrogen™) (Table S1). The PCR mix included 1 x HotStar Taq DNA polymerase buffer, 200 µM of each dNTP, 200 nM of each primer and 2 IU of HotStar Taq DNA polymerase (Table S1). Cycling conditions included an initial denaturation step performed for 10 min at 95°C, following 50 cycles of 30 s denaturation at 95°C, 30 s annealing at 60°C and 30 s elongation at 72°C. 10 µL of PCR product was purified and prepared for Pyrosequencing. DNA methylation analysis was performed using PyroMark Gold SQA Q 96 Kit (QIAGEN) on a PyroMark Q96 MD and analysed with

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the PyroMark CpG software (QIAGEN). The method depends on amplification of the 308 bp fragment and pyrosequencing of a short segment comprising 7 CpG positions in the region on chromosome 6 (Chr 6), spanning positions 11,044,661; 11,044,655; 11,044,647; 11,044,644; 11,044,642; 11,044;640; and 11,044;634.[16] Per cent methylation at each CpG locus was calculated using the Pyro Q CpG software. The *ELOLV2* single locus age prediction model has been reported by several studies.[16–20]

ELOVL2 methylation of a reference DNA sample was measured on separate days to control for between-day variations. The between-day coefficient of variation was less the 5% for all CpG loci (Table S2).

2.6 | Statistical analyses

The descriptive results are shown as median (5th and 95th percentiles) or mean (standard deviation, SD) for continuous variables and absolute (*n*) and relative frequencies (%) for categorical variables. We used One-Sample Kolmogorov–Smirnov test to study if the methylation of the 7 CpG cites of ELOVL2-promoter are normally distributed. We additionally used Q-Q plots to judge the distribution. Only ELOVL2-2 and ELOVL2-5 formally fulfilled the normal distribution assumption (*p* values for One-Sample Kolmogorov–Smirnov >0.05). The remaining variables of ELOVL2-methylation were $\log_{10}$-transformed to check if the distribution improved. However, the non $\log_{10}$-transformed data were found to be closer to normal distribution than the log-transformed values (both on One-Sample Kolmogorov–Smirnov and Q-Q plots). Thus, the native methylation results were used for the data analysis.

Pearson test was used to study the binary correlations between ELOVL2-methylation and continuous variables such as age, blood concentrations of lipids, blood pressure or BMI. Pearson test was repeated after adjusting for age. Additionally, we studied the associations between ELOVL2-methylation and classical cardiovascular and renal risk factors at baseline using multivariate linear regression analyses with separate models for each ELOVL2-methylation cite that was entered as dependent variable and the risk factors as independent variables.

The one-way analysis of variance (ANOVA) test was used to compare the methylation levels between independent groups. When ANOVA test was significant, subgroup analysis was performed using the post hoc Tamhane-T2 test. Moreover, we used ANOVA with analysis of covariance (ANCOVA) to study whether each of the ELOVL2-methylation levels (dependent variable) differ according to the CKD stage (fixed factor) after adjusting for age (covariate). The interaction between CKD stage and age was studied (whether the association between ELOVL2 methylation and CDK stage differed by age).

2.7 | The risk of future clinical outcomes

Cox-regression was applied to study the hazard ratio (HR) and the 95% confidence intervals (95% CI) of the primary clinical outcomes in relation to baseline ELOVL2-methylation. During the mean follow-up time of 3.9 years, renal outcomes occurred in 64 patients (i.e. halving of eGFR from baseline, transfer to dialysis, and all-cause mortality combined) and cardiovascular outcomes occurred in 77 patients (cardiovascular event and all-cause mortality combined). The crude and the adjusted HR (95% CI) are presented. Loss to follow-up during this 3.9 years period was due to death.

2.8 | Covariates

We defined a set of covariates based on their known association with DNA-methylation and the clinical outcomes of CKD in the same time, or their association with the clinical outcomes of CKD only. Cox-regression analyses were run for each ELOVL2-methylation cite separately. Model 1 is the crude or unadjusted model. Model 2 was adjusted for age, CRP, plasma cholesterol, and neutrophils/lymphocytes ratio (as continuous variables) and sex (M, F) and smoking (yes, no) as categorical variables. Model 3 was adjusted for the same covariates in Model 2 in addition to eGFR as a continuous variable. The adjustment for the ratio of neutrophils to leucocytes aimed to control for the effect of cellular heterogeneity on DNA-methylation.[21] Adjusted data analyses were run on available data ($n = 349$) as one CRP value was missing. Sensitivity analysis included further adjustments for blood pressure (systolic and diastolic) and BMI as continuous variables and diabetes (yes, no). We also run separate sensitivity analysis adjusting for plasma triglycerides or LDL-cholesterol concentrations instead of total cholesterol.

The statistical analyses were conducted using version 29 of IBM® SPSS® Statistics package (SPSS Inc.). *p*-values ≤0.05 were considered statistically significant.

3 | RESULTS

3.1 | Baseline characteristics of the study cohort

The clinical characteristics of the study participants are shown in Table 1 [Median (5–95th) age = 67.9 (44.0–81.7);

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TABLE 1 Demographic and clinical characteristics of 350 participants with chronic kidney disease stage G2 to G4 at baseline visit.

Characteristics	Median (5th–95th) or *n* (%)
Age, years	67.9 (44.0–81.7)
Women, *n* (%)	162 (46)
Smoker, *n* (%)	30 (8.5)
BMI, kg/m^2	29.9 (22.1–40.7)
Systolic blood pressure, mmHg	147 (118–197)
Diastolic blood pressure, mmHg	84 (65–106)
Blood markers	
Plasma glucose, mg/dL	111 (87–187)
Plasma cholesterol, mmol/L	4.69 (3.16–6.84)
Plasma triglyceride, mmol/L	1.45 (0.66–3.58)
Plasma LDL-cholesterol, mmol/L	2.74 (1.33–4.61)
Plasma HDL-cholesterol, mmol/L	1.32 (0.83–2.21)
Apolipoprotein A-1, mg/dL	164 (120–225)
Apolipoprotein B, mg/dL	94 (55–144)
Plasma troponin, pg/mL	11.0 (2.9–50.0)
Plasma NT-proBNP, pg/mL	202.9 (26.7–4651.1)
Plasma creatinine, mmol/L	117.6 (76.6–224.1)
Plasma cystatin C, mg/L	1.45 (0.95–2.76)
Plasma CRP, mg/L	2.6 (0.5–15.9)
Haemoglobin, g/dL	13.5 (10.9–15.9)
Lymphocytes, %	26.0 (13.0–41.4)
Neutrophils, %	62.0 (47.0–77.0)
Neutrophils/Lymphocytes ratio	3.0 (1.0–4.0)
Clinical conditions and drugs	
Main renal diseases, *n* (%)	
Nephrosclerotic vascular diseases	163 (46.3)
Diabetic glomerosclerosis	28 (8.0)
Cystic diseases (autosomal-dominant polycystic kidney disease, other cystic diseases)	15 (4.3)
Vascular diseases (renal artery stenosis)	13 (3.7)
Mesangial proliferative glomerulonephritis	11 (3.1)
Post-transplant	8 (2.3)
Unknown	47 (13.4)
Others (e.g. non-inflammatory glomerular disease, reflux nephropathy, microangiopathy, etc.)	67 (19.0)
Confirmed renal biopsy, *n* (%)	28 (8.0)
Any coronary artery disease, *n* (%)	78 (22.2)
Stroke, *n* (%)	28 (8.0)
Cerebrovascular disease, *n* (%)	39 (11.1)
Any cardiovascular disease, *n* (%)	114 (32.4)
Diabetes mellitus, *n* (%)	139 (39.5)
Using oral anti-diabetes drugs, *n* (%)	82 (23.3)
Insulin therapy, *n* (%)	52 (14.8)
Statin use, *n* (%)	201 (57.1)
Use of beta blockers, *n* (%)	244 (69.3)
Use of ACE inhibitors, *n* (%)	116 (33)
Loop diuretics (e.g. furosemid, piretanid and torasemid), *n* (%)	145 (41.2)
Thiazide diuretics, *n* (%)	176 (50.0)
Any vitamin D treatment, *n* (%)	160 (45.5)
Native vitamin D supplementation (ergocalciferol, cholecalciferol or calcifediol), *n* (%)	157 (44.6)
Chronic kidney disease stage[a], *n* (%)	
G2 (eGFR min–max. 60.2–88.4 mL/min/1.73 m^2)	81 (23.1)
G3a (eGFR min–max. 45.1–59.9 mL/min/1.73 m^2)	126 (36.0)
G3b (eGFR min–max. 30.0–44.8 mL/min/1.73 m^2)	94 (26.9)
G4 (eGFR min–max. 17.2–29.8 mL/min/1.73 m^2)	49 (14.0)

[a]Chronic kidney disease stage according to KDIGO.

Abbreviations: ACE, angiotensin-converting enzyme; BMI, body mass index; CKD, chronic kidney disease; CRP, C-reactive protein; HDL, high density lipoprotein; LDL, low density lipoprotein; NT-proBNP, amino-terminal pro-brain natriuretic peptide.

$n=350$, 46% women]. At the baseline visit, 81 patients (23.0%) were classified in CKD KDIGO stage G2, 126 (36.0%) in stage G3a, 94 (26.9%) in stage G3b and 49 (13.9%) as stage G4. Common morbidities were; diabetes mellitus in 139 patients (39.5%), cardiovascular disease in 114 patients (32.4%), coronary artery diseases in 78 patients (22.2%), stroke in 28 patients (8.0%) and cerebrovascular disease in 39 patients (11.1%).

3.2 | ELOVL2 CpGs methylation, age and chronic kidney disease stage

The results of the methylation of the seven ELOVL2 CpG-positions are shown in Table 2. The methylation of the seven CpGs showed direct and strong correlations with age (i.e. the CpGs where hypermethylated in older persons). The correlation coefficients ranged between 0.67 and 0.87 (all $p < 0.001$, Pearson test) (Figure S1).

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The ELOVL2-methylation levels differed according to the CKD stages. ELOVL2 CpGs methylation was significantly lower in patients with CKD G2 compared to those with CKD G3a, G3b and CKD G4 (Table 3). Patients with CKD G2 were younger than the other CKD groups. Univariate ANCOVA models showed that ELOVL2 methylation at positions 4, 5 and 6 did not differ between the CKD stages after adjusting for age (Table 3). The differences in ELOVL2 methylation at positions 1, 2, 3 and 7 between the CKD stages showed significant interactions with age (Table 3), thus differences in age between the CKD groups were likely to explain the between-CKD stage differences in ELOVL2-methylation.

TABLE 2 ELOVL2 promoter methylation (%) among 350 patients with CKD stage G2 to G4 and available DNA samples from baseline visit.

Position no.	Position ID	Median (5th–95th)
1	chr6:11,044,661	48.0 (36.9–59.2)
2	chr6:11,044,655	45.6 (32.3–57.3)
3	chr6:11,044,647	81.4 (70.9–88.1)
4	chr6:11,044,644	62.5 (49.5–72.7)
5	chr6:11,044,642	41.3 (29.1–56.1)
6	chr6:11,044,640	91.2 (75.3–100.0)
7	chr6:11,044,634	93.1 (81.7–98.8)

Abbreviation: ELOVL2, Elongation Of Very Long Chain Fatty Acids-Like 2.

3.3 | Cross sectional associations between ELOVL2-methylation and cardiovascular risk factors

After adjusting for age, ELOVL2-CPGs methylation showed only week correlations with BMI (Pearson correlation coefficient $r=0.14$, $p=0.017$ only with ELOVL2-5) and CRP ($r=-0.15$, $p=0.012$, only with ELOVL2-2). In the age-adjusted analyses, there were no significant correlations between ELOVL2-methylation and plasma concentrations of plasma lipids, cystatin C, NT-pro-PNP, apolipoproteins (A1 and B) or plasma glucose. Similarly, there was no correlation between ELOVL2-methylation

TABLE 3 ELOVL2-methylation at seven CpG positions in patients with CKD stages G2–G4 at baseline visit according to the stage of chronic kidney function.

	CKD G2	CKD G3a	CKD G3b	CKD G4	*p* value*	*p* adjusted for age**	*p* for interactions*** CKD stage × age
Number (*n* females)	*N* = 81 (F; 32)	*N* = 126 (F; 64)	*N* = 94 (F; 41)	*N* = 49 (F; 23)	–	–	–
eGFR, mL/min/1.73 m^2	69.2 (7.1)	51.7 (4.1)	37.9 (4.3)	23.9 (3.7)	–	–	–
Age, years	59 (13)	66 (12)	70 (11)	70 (10)	<0.001	–	–
ELOVL2 methylation, %							
Position 1-chr6:11,044,661	45.9 (7.3)	48.4 (7.0)	49.5 (7.7)[a]	49.1 (5.7)[a]	0.007	0.045	0.022
Position 2-chr6:11,044,655	43.0 (8.0)	45.9 (7.1)[a]	47.0 (7.4)[a]	45.4 (6.4)	0.003	0.005	<0.001
Position 3-chr6:11,044,647	78.2 (6.4)	80.9 (5.3)[a]	81.8 (5.0)[a]	81.8 (4.0)[a]	<0.001	<0.001	<0.001
Position 4-chr6:11,044,644	59.3 (7.7)	62.0 (7.0)	63.6 (7.4)[a]	63.9 (5.9)[a]	<0.001	0.350	0.260
Position 5-chr6:11,044,642	38.5 (8.3)	41.5 (7.1)[a]	43.8 (9.1)[a]	43.6 (6.7)[a]	<0.001	0.197	0.182
Position 6-chr6:11,044,640	86.7 (8.6)	90.1 (8.5)[a]	90.3 (8.8)[a]	91.7 (8.7)[a]	0.006	0.529	0.395
Position 7-chr6:11,044,634	89.3 (6.4)	92.2 (5.4)[a]	92.7 (11.0)[a]	93.0 (4.2)[a]	<0.001	0.020	0.022

Note: Data are shown as mean (SD) unless otherwise specified. The difference in each CpG-methylation cite between the CKD groups was studied using a univariate ANOVA test.

[a]Significantly different from CKD G2 (ANOVA and post hoc Tamhane-Test).

*Overall unadjusted p values of the comparisons of ELOVL2-CpGs methylation between the CKD subgroups (ANOVA); ***p* values for the comparisons of ELOVL2-CpGs methylation between the CKD subgroups after adjusting for age (covariate) according to univariate ANCOVA; ****p* values for the interaction between the CKD stage and age.

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and blood pressure (systolic and diastolic). Higher plasma glucose concentrations showed weak inverse associations with ELOVL2-methylation at Positions 1 and 2 (Pearson correlation coefficient $r = -0.11$) after adjustment for age.

We used multivariate linear regression analysis to investigate whether ELOVL2-methylation is associated with age, BMI, blood pressure (systolic and diastolic), plasma concentrations of cholesterol, cystatin C, and CRP, the ratio of neutrophils to lymphocytes, the presence of diabetes, sex (M or F) and smoking (yes or no) (Table 4). Age was a significant positive determinant of ELOVL2-methylation at all CpG positions. Moreover, being a non-smoker (a negative determinant of ELOVL2-methylation) and neutrophils to lymphocytes ratio (a negative determinant of the ELOVL2-methylation) were significant determinants of the methylation at different CpG positions (Table 4). Higher neutrophils to lymphocytes ratio had quantitatively the largest beta coefficient for the association with lower ELOVL2-methylation (Table 4).

3.4 | Baseline ELOVL2 methylation and the risk of renal and cardiovascular outcomes

During the follow-up time of 3.9 years, 64 renal endpoint and 77 vascular endpoints occurred. The crude and adjusted HR and 95% CI for the renal or vascular outcomes in relation to baseline ELOVL2-methylation are shown in Table 5. We found no significant association between methylation of any of the seven ELOVL2 positions and the risk of renal or cardiovascular events in the crude models or after adjustment for the covariates (Table 5).

4 | DISCUSSION

The clinical value of predicting premature ageing and the benefit of anti-ageing strategies are debatable. The role of ELOVL2-methylation as a marker or pathological factor in ageing is intriguing as it can in theory enable disease prediction and prevention. The present explorative study among patients with CKD stage G2–G4 and a wide age range has shown that ELOVL2-hypermethylation in whole blood is strongly associated with higher age. Higher ELOVL2-methylation was observed in patients with CKD stage G3a, G3b and G4 compared those with CKD stage G2. However, the association was largely explained by age differences between the CKD subgroups. In a cross-sectional analysis of baseline data, we found that the between-subject variations in ELOVL2-methylation were explained by age (associated with ELOVL2-hypermethylation), smoking (partly associated with ELOVL2-hypermethylation) and

TABLE 4 Multivariate linear regression models showing the associations between ELOVL2-methylation and cardiovascular risk factors.

	ELOVL2-1	ELOVL2-2	ELOVL2-3	ELOVL2-4	ELOVL2-5	ELOVL2-6	ELOVL2-7
Age, years	0.41 (0.36, 0.47)	0.45 (0.39, 0.50)	0.37 (0.34, 0.40)	0.47 (0.43, 0.52)	0.49 (0.43, 0.54)	0.58 (0.53, 0.63)	0.39 (0.36, 0.43)
Non-smokers vs. smokers	−2.14 (−4.22, −0.06)	−1.74 (−3.76, 0.29)	−1.36 (−2.64, −0.08)	−1.57 (−3.19, 0.04)	−1.95 (−4.12, 0.204)	−2.16 (−4.19, −0.14)	−1.13 (−2.42, −0.17)
Neutrophils/lymphocytes ratio	−1.19 (−1.65, −0.73)	−1.44 (−1.89, −0.99)	−0.49 (−0.78, −0.22)	−0.93 (−1.29, −0.57)	−1.19 (−1.67, −0.77)	−0.59 (−1.03, −0.14)	−0.24 (−0.52, −0.05)
Plasma cholesterol, mmol/L			−0.42 (−0.75, −0.09)				
BMI, kg/m^2					−0.143 (−0.25, −0.04)		

Note: Data are the regression beta coefficient and (95% confidence intervals). Each model included one methylation cite at a time as dependent variable. The following variables were entered as independent variables: age, BMI, blood pressure (systolic and diastolic), plasma concentrations of cholesterol, cystatin C, and CRP, the ratio of neutrophils to lymphocytes (as continuous variables); and the presence of diabetes, sex (M or F) and smoking (yes or no) were entered as categorical variables.

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TABLE 5 Hazard ratio (HR) and 95% confidence intervals (95% CI) for the primary clinical outcomes among patients with CKD stage G2–G4.

Duration of follow-up time, years; mean (SD); [range]	Renal event or death, $N = 64$			Cardiovascular event or death, $N = 77$		
	3.9 (1.8); [0.2, 7.2]			**3.9 (1.9); [0.2, 7.0]**		
ELOVL2-methylation cites	Model 1 (Crude)	Model 2	Model 3	Model 1 (Crude)	Model 2	Model 3
Position 1- chr6:11,044,661	1.03 (1.03, 1.07)	1.00 (0.95, 1.04)	1.02 (0.97, 1.06)	1.03 (1.00, 1.06)	0.98 (0.94, 1.02)	0.99 (0.95, 1.03)
Position 2- chr6:11,044,655	1.03 (1.00, 1.06)	1.00 (0.95, 1.04)	1.01 (0.98, 1.06)	1.02 (0.99, 1.05)	0.98 (0.94, 1.01)	0.99 (0.95, 1.02)
Position 3- chr6:11,044,647	1.06 (1.01, 1.11)	1.00 (0.93, 1.08)	1.00 (0.94, 1.07)	1.05 (1.00, 1.10)	0.94 (0.87, 1.01)	0.94 (0.88, 1.01)
Position 4- chr6:11,044,644	1.04 (1.00, 1.08)	0.98 (0.92, 1.05)	0.98 (0.93, 1.04)	1.04 (1.01, 1.07)	0.96 (0.91, 1.02)	0.96 (0.91, 1.02)
Position 5- chr6:11,044,642	1.04 (1.01, 1.07)	1.01 (0.97, 1.06)	1.00 (0.96, 1.04)	1.05 (1.02, 1.08)	1.01 (0.98, 1.05)	1.01 (0.97, 1.05)
Position 6- chr6:11,044,640	1.03 (1.00, 1.06)	0.96 (0.92, 1.00)	0.97 (0.93, 1.00)	1.05 (1.01, 1.08)	0.98 (0.94, 1.02)	0.99 (0.95, 1.02)
Position 7- chr6:11,044,634	1.05 (1.00, 1.10)	0.95 (0.88, 1.03)	0.97 (0.90, 1.05)	1.06 (1.01, 1.11)	0.97 (0.90, 1.04)	0.98 (0.91, 1.05)

Note: Cox regression analyses were run for each ELOVL2 methylation cites separately. Model 1 is the crude (unadjusted) model; Model 2 is adjusted for age, CRP, plasma cholesterol and neutrophils/lymphocytes ratio (as continuous variables) and sex and smoking as categorical variables; Model 3 is adjusted for the same covariates in Model 2 in addition to eGFR (as a continuous variable). ELOVL2 methylation was used as native values (not log-transformed).

higher neutrophils/lymphocytes ratio (associated with lower ELOVL2-methylation). In the 3.9-years longitudinal study, whole blood ELOVL2-methylation was not independently associated with future deterioration of renal function combined with all-cause mortality, neither it was associated with combined cardiovascular events and all-cause mortality.

Our results on the lack of associations between whole blood ELOVL2-methylation and clinical outcomes are in line with a previous study on ELOVL2 gene methylation among elderly people with a mean age of 94 years.[2] Whereas, other studies found that some epigenetic age prediction models (i.e. Horvath's and Hannum's) can predict mortality[22,23] or longevity.[24] It is not clear whether these inconsistent results are due to the model used to predict epigenetic age, the study cohort or the data analysis. Obviously, residual confounding is likely. While some studies adjusted for several confounders,[22] others adjusted only for age, sex and blood count.[24] Studies focusing on very old persons may be subject to selection bias due to premature censoring of people with unknown methylation levels. Moreover, studies not adjusting for different types of cells in whole blood may be subject to bias due to differential methylation of the genes according to their cell origin. Whole blood is a heterogeneous mix of white cells that differ in their ELOVL2 methylation thus, adjustment for cell heterogeneity has been recommended when whole blood methylation is used as an ageing marker.[25] Interpretation of DNA-methylation profile from a mix of different cell pattern could be influenced by the proportion of white blood cells in some inflammatory diseases or cancer.

ELOVL2-hypermethylation with age is assumed to cause a lower translation and activity of this elongase enzyme. Experimental studies in mice and zebrafish have shown that inactivation of Elvol2 (i.e. knockout or down regulation) caused accelerated aging phenotype.[7,26] However, only partial improvement was seen after dietary supplementation of PUFAs in Elovl2- knockout mice,[8] suggesting that Elvol2 may affect pathways other than PUFAs. Results of studies conducted by Liu et al. have suggested that DNA-methylation may play an intermediary role rather than a causal role in the relationship between the ELOVL2 gene and clinical outcomes.[25] Moreover, three ELOVL2 genetic variants were found to be protective factors against high cholesterol and glucose, insulin resistance and high body fat content.[27] Other SNPs in ELOVL2 gene were associated with risk factors of cardiovascular diseases such as total- and LDL-cholesterol, insulin and BMI.[27] Therefore, hypermethylation of ELOVL2 gene in whole blood does not necessarily mirror a risk factor or a mechanism of disease. Moreover, methylation of ELOVL2 in persons carrying high-risk SNPs may alter the overall risk.

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ELOVL2-methylation showed positive linear association with age in previous studies as in the present one.[16,19] Although ELOVL2-methylation in most of the investigated tissues (including several types of blood cells) showed a strong correlation with age,[2] the slope of the association varied between tissues. Ronn et al., suggested that variations in methylation of DNA isolated from blood cells can mirror epigenetic signatures in adipose tissue and potentially be used as epigenetic biomarkers.[28] However, it is not clear whether ELOVL2-methylation in whole blood may be representative for ELOVL2-methylation in the kidney, the heart or the vascular system.[2]

The main limitation of this study is the rather short follow-up time that may not allow detecting a large number of events. However, the general risk of future morbidities and mortality is high is patients with CKD which could otherwise takes decades to detect in a cohort of healthy population. Moreover, the follow-up time in this study is similar to previous studies such as The Chronic Renal Insufficiency Cohort (CRIC) Study with a median follow-up time of 3.7 years.[29] Finally, we have no reference group of completely healthy population to compare with. However, the large number of patients, the wide range of age and the detailed information on their health condition and risk factors are important strengths of the present study.

Taken together, the present study in patients with CKD stage G2 through G4 confirmed that ELOVL2-hypermethylation is strongly associated with age. ELOVL2-methylation was not independently associated with renal or cardiovascular risk factors and was not a significant risk factor for future deterioration of renal function, cardiovascular events and all-cause mortality. The results are likely to be representative of this high risk population, but the association between initial ELOVL2 methylation in otherwise healthy population and future CVD or renal dysfunction could differ from that in the present study. Our results suggest that ELOVL2-methylation is not likely to be an independent predictor of underlying disease mechanisms of future adverse health events. ELOVL2-methylation could be an epiphenomenon of a systemic aging process that can itself cause diseases. Polymorphisms in ELOVL2 gene or other related genes might be one of these upstream processes that deserve further investigation.

AUTHOR CONTRIBUTIONS

Rima Obeid, planned the present study, conducted data analyses and drafted the manuscript. Insa E. Emrich and Adam M. Zawada, collected biosamples and clinial data. Gunnar Henrik Heine and Danilo Fliser planned and supervised the original CARE FOR HOMe study. Jürgen Geisel, planned and supervised the present study. Patricia Rickens and Marion Bodis, conducted measurements of ELOVL2 methylation.

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CONFLICT OF INTEREST STATEMENT

RO, PR, GHH, IEE, DF, MB and JG have no conflict of interest. AMZ is currently full-time employee of Fresenius Medical Care, while the current work was performed under the former affiliation (Saarland University Medical Center).

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REFERENCES

1. Huan T, Nguyen S, Colicino E, et al. Integrative analysis of clinical and epigenetic biomarkers of mortality. *Aging Cell*. 2022;21(6):e13608.
2. Bacalini MG, Deelen J, Pirazzini C, et al. Systemic age-associated DNA hypermethylation of ELOVL2 gene: In vivo and In vitro evidences of a cell replication process. *J Gerontol A Biol Sci Med Sci*. 2017;72(8):1015-1023.
3. Garagnani P, Bacalini MG, Pirazzini C, et al. Methylation of ELOVL2 gene as a new epigenetic marker of age. *Aging Cell*. 2012;11(6):1132-1134.
4. Mansego ML, Milagro FI, Zulet MA, Moreno-Aliaga MJ, Martinez JA. Differential DNA methylation in relation to age and health risks of obesity. *Int J Mol Sci*. 2015;16(8):16816-16832.
5. Jakobsson A, Westerberg R, Jacobsson A. Fatty acid elongases in mammals: their regulation and roles in metabolism. *Prog Lipid Res*. 2006;45(3):237-249.
6. Chao DL, Skowronska-Krawczyk D. ELOVL2: not just a biomarker of aging. *Transl Med Aging*. 2020;4:78-80.
7. Chen D, Chao DL, Rocha L, et al. The lipid elongation enzyme ELOVL2 is a molecular regulator of aging in the retina. *Aging Cell*. 2020;19(2):e13100.
8. Li X, Wang J, Wang L, et al. Lipid metabolism dysfunction induced by age-dependent DNA methylation accelerates aging. *Signal Transduct Target Ther*. 2022;7(1):162.
9. Zadravec D, Tvrdik P, Guillou H, et al. ELOVL2 controls the level of n-6 28:5 and 30:5 fatty acids in testis, a prerequisite for male fertility and sperm maturation in mice. *J Lipid Res*. 2011;52(2):245-255.
10. Cruciani-Guglielmacci C, Bellini L, Denom J, et al. Molecular phenotyping of multiple mouse strains under metabolic challenge uncovers a role for Elovl2 in glucose-induced insulin secretion. *Mol Metab*. 2017;6(4):340-351.

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11. Hannum G, Guinney J, Zhao L, et al. Genome-wide methylation profiles reveal quantitative views of human aging rates. *Mol Cell*. 2013;49(2):359-367.
12. Marioni RE, Shah S, McRae AF, et al. The epigenetic clock is correlated with physical and cognitive fitness in the Lothian Birth Cohort 1936. *Int J Epidemiol*. 2015;44(4):1388-1396.
13. Marioni RE, Harris SE, Shah S, et al. The epigenetic clock and telomere length are independently associated with chronological age and mortality. *Int J Epidemiol*. 2018;45(2):424-432.
14. Untersteller K, Seiler-Mussler S, Mallamaci F, et al. Validation of echocardiographic criteria for the clinical diagnosis of heart failure in chronic kidney disease. *Nephrol Dial Transplant*. 2018;33(4):653-660.
15. Emrich IE, Zawada AM, Martens-Lobenhoffer J, et al. Symmetric dimethylarginine (SDMA) outperforms asymmetric dimethylarginine (ADMA) and other methylarginines as predictor of renal and cardiovascular outcome in non-dialysis chronic kidney disease. *Clin Res Cardiol*. 2018;107(3):201-213.
16. Zbiec-Piekarska R, Spolnicka M, Kupiec T, et al. Examination of DNA methylation status of the ELOVL2 marker may be useful for human age prediction in forensic science. *Forensic Sci Int Genet*. 2015;14:161-167.
17. Paparazzo E, Lagani V, Geracitano S, et al. An ELOVL2-based epigenetic clock for forensic age prediction: a systematic review. *Int J Mol Sci*. 2023;24(3):2254.
18. Pfeifer M, Bajanowski T, Helmus J, Poetsch M. Inter-laboratory adaption of age estimation models by DNA methylation analysis-problems and solutions. *Int J Leg Med*. 2020;134(3):953-961.
19. Bekaert B, Kamalandua A, Zapico SC, Van de Voorde W. Improved age determination of blood and teeth samples using a selected set of DNA methylation markers. *Epigenetics*. 2015;10(10):922-930.
20. Daunay A, Baudrin LG, Deleuze JF, How-Kit A. Evaluation of six blood-based age prediction models using DNA methylation analysis by pyrosequencing. *Sci Rep*. 2019;9(1):8862.
21. Amiri RM, Mousavi SF, Salek AS, et al. Prediction of biological age and evaluation of genome-wide dynamic methylomic changes throughout human aging. *G3 (Bethesda)*. 2021;11(7):1-12.
22. Marioni RE, Shah S, McRae AF, et al. DNA methylation age of blood predicts all-cause mortality in later life. *Genome Biol*. 2015;16(1):25.
23. Christiansen L, Lenart A, Tan Q, et al. DNA methylation age is associated with mortality in a longitudinal Danish twin study. *Aging Cell*. 2016;15(1):149-154.
24. Horvath S, Pirazzini C, Bacalini MG, et al. Decreased epigenetic age of PBMCs from Italian semi-supercentenarians and their offspring. *Aging (Albany NY)*. 2015;7(12):1159-1170.
25. Liu Y, Aryee MJ, Padyukov L, et al. Epigenome-wide association data implicate DNA methylation as an intermediary of genetic risk in rheumatoid arthritis. *Nat Biotechnol*. 2013;31(2):142-147.
26. Dasyani M, Gao F, Xu Q, et al. Elovl2 is required for robust visual function in zebrafish. *Cell*. 2020;9(12):1.
27. Maycotte-Cervantes ML, Aguilar-Galarza A, Anaya-Loyola MA, et al. Influence of single nucleotide polymorphisms of ELOVL on biomarkers of metabolic alterations in the Mexican population. *Nutrients*. 2020;12(11):3389.
28. Ronn T, Volkov P, Gillberg L, et al. Impact of age, BMI and HbA1c levels on the genome-wide DNA methylation and mRNA expression patterns in human adipose tissue and identification of epigenetic biomarkers in blood. *Hum Mol Genet*. 2015;24(13):3792-3813.
29. Scialla JJ, Xie H, Rahman M, et al. Fibroblast growth factor-23 and cardiovascular events in CKD. *J Am Soc Nephrol*. 2014;25(2):349-360.

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