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ARTICLE

Mortality risk in men is associated with a common mutation in the methylene-tetrahydrofolate reductase gene (MTHFR)

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An elevated level of homocysteine in plasma is associated with the occurrence of cardiovascular disease. A common *ala*-to-*val* mutation in the methylenetetrahydrofolate reductase gene (MTHFR) is associated with an elevated level of plasma homocysteine. We studied the possible detrimental effects of the MTHFR mutation on mortality. Within a population-based study in the city of Leiden, the Netherlands, we first compared the MTHFR genotype distribution among 365 elderly subjects aged 85 years and over born in Leiden, and 250 young subjects aged 18 to 40 years whose families originated from the same geographical region. Second, the complete cohort of 666 subjects aged 85 years and over was followed over a period of 10 years for all-cause and cause-specific mortality and stratified according to MTHFR genotype. The frequency of the MTHFR mutation was significantly lower in the elderly than in the young (0.30 and 0.36, respectively; $P = 0.03$). The difference in genotype distribution was only present in men. The estimated mortality risk up to 85 years in men carrying the *val*/*val* genotype was 3.7 (95% confidence interval (CI), 1.3–10.9). Over the age of 85, mortality in men with the *val*/*val* genotype was increased 2.0-fold (95% CI, 1.1–3.9) and appeared to be attributable to cancer rather than cardiovascular causes of death. Among women aged 85 years and over, no deleterious effect of the MTHFR mutation was observed. In conclusion, the MTHFR mutation is associated with increased mortality in men in middle and old age, but not in women.

Keywords: methylenetetrahydrofolate reductase; mortality; cardiovascular diseases; cancer; homocysteine; longevity genetics; ageing

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Introduction

An elevated level of homocysteine in plasma is associated with the occurrence of cardiovascular disease[1] and increased mortality in patients with coronary artery disease.[2] A recent meta-analysis estimated that every 5 μmol/l increment in plasma homocysteine increases the risk of coronary heart disease by 60% for men and 80% for women.[1] Homocysteine is formed when the methyl-group of methionine is transferred to DNA, proteins or other molecules. The basal level of plasma homocysteine is mainly determined by the remethylation of homocysteine to methionine.[3,4] This reaction is regulated by the enzyme methylenetetrahydrofolate reductase (MTHFR)[3] which converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. The methionine/homocysteine metabolism is disrupted by deficiencies in the essential coenzymes vitamin B6, B12 and folate, and by homozygosity for rare mutations in the genes encoding MTHFR and cystathionine β synthase.[5,6] These genetic defects give rise to greatly elevated homocysteine levels in plasma and result in mental retardation, bone malformations and premature atherosclerotic disease.[5,6]

Recently, a common C_{677}-to-T (*ala*-to-*val*) mutation in the MTHFR gene was identified[7] which leads to a less severe disturbance of the methionine/homocysteine metabolism. About half the general population carries at least one mutated allele and the frequency of the homozygous mutated genotype (*val*/*val*) varies from 8% to 18% depending on the population.[8–19] The MTHFR mutation was shown to render the enzyme thermolabile, and homozygotes and heterozygotes had about a 70% and 35% reduced MTHFR activity, respectively.[7] Furthermore, homozygosity for the mutation is associated with elevated levels of homocysteine in plasma.[7,9,11,15–17,19–21] This association is dependent on age[15,17] and nutrition. Plasma homocysteine levels are predominantly elevated among carriers of the *val*/*val* genotype who have a low level of plasma folate.[9,11,15] Moreover, it was shown that especially in *val*/*val* carriers the level of plasma homocysteine was lowered by folic acid supplementation.[22] In various reports the MTHFR mutation has been implicated in the risk of cardiovascular disease[4,10,14–16,18,19,21,23–28] and cancer[12,29] but the data are equivocal.

To explore the possible detrimental effects of a disturbed methionine/homocysteine metabolism, we studied the association between the MTHFR mutation and mortality, nested in a population-based study of subjects aged 85 years and over (Leiden 85-plus Study). This was done (i) in a cross-sectional analysis comparing the occurrence of the MTHFR mutation between subjects aged 85 years and over and young subjects aged 18–40 years whose families originated from the same geographical region as the elderly subjects, and (ii), prospectively, over a 10-year follow-up period in the entire elderly cohort. The follow-up study included the analysis of cause-specific mortality risks.

Materials and Methods

The Leiden 85-plus Study is a population-based study in which all inhabitants of Leiden aged 85 years and over were invited to take part.[30] Of a total of 1258 eligible subjects, 221 died before enrolment which lasted from 1 December 1986 to 1 March 1988. Of the 1037 remaining subjects, 977 (94%) participated and were medically interviewed at home.[31] After the exclusion of subjects with a non-Dutch (n = 29) or unknown (n = 69) place of birth, sufficient cell material was available from 666 (188 men, 478 women) subjects for the present genetic study. DNA was extracted[32] and MTHFR genotypes were determined by the PCR-amplification of a 198 bp fragment containing the C_{677}-to-T transition, followed by digestion with *Hin*fI as previously described.[7] The MTHFR genotype was independently assessed by two observers. As a standard laboratory procedure a randomly chosen 10% of the samples was reamplified. No genotyping errors were observed. The study was approved by the Medical Ethics Committee of Leiden University and informed consent was obtained from all participants.

Cross-sectional Analysis

MTHFR genotype distributions were compared in elderly subjects aged 85 years and over and young controls. The subjects in the elderly population are survivors of a cohort born between 1887–1901. To avoid false associations with the MTHFR mutation due to differences in geographical origin rather than age, the cross-sectional comparison of elderly and young subjects accounted for local variations in MTHFR genotype distribution that may have existed in the past. Subjects aged 85 years and over who were born in Leiden (n = 365; 56%) were compared with a control population which consisted of 250 (139 men, 111 women) blood donors aged 18–40 years of Dutch descent with either one parent born in Leiden and the other within 12 km of Leiden, or with two Leiden-born parents. Information regarding the birthplace of their grandparents was obtained from a written questionnaire. If a specific Leiden MTHFR genotype distribution had existed in the past, the genotype distribution in young controls would have increasingly deviated, the greater the number of their Leiden-born grandparents. The upper age limit of the young controls was chosen since selection against genotypes contributing to population mortality was not expected to occur before the age of 40 years.

Prospective Follow-up Study

All participants in the Leiden 85-plus Study were followed up for mortality until 1 October 1996. Among the 666 subjects of the cohort studied, two were lost to follow-up. Primary causes of death were assessed by linking the death certificate

numbers, obtained from civic registers, to the causes of death as recorded by the Dutch Central Bureau of Statistics. Causes of death were classified according to the ninth revision of the *International Classification of Diseases (ICD-9)*.[33] Death certificates from 1996, coded according to the tenth revision of the *International Classification of Diseases*, were recoded according to the ninth revision. *ICD-9* codes were reviewed and each code was categorised for cardiovascular disease (*ICD-9* 390–459), cancer (*ICD-9* 140-239) and all causes (*ICD-9* 000–999). Death from infection was coded as previously described.[34]

Statistical Analysis

Differences in baseline characteristics were tested for significance with the χ^2 test for categorical and Student's *t*-test for continuous variables. In the cross-sectional analysis, distributions of alleles and genotypes were compared by the χ^2 test, and mortality risks and 95% CIs were estimated using the exposure odds ratio. Mantel's extension of the Mantel-Haenszel test was used to test for trend in stratified analyses.[35] In the follow-up study, survival times for subjects were computed from the date of the home visit to the date of one of the following events: death from a specific cause, death from any cause, or 1 October 1996. Survival was estimated using the Kaplan-Meier product limit method and compared with the log-rank test. Adjusted mortality risks and 95% CIs were estimated with Cox proportional hazards models. Causes of death were assumed to be independent. *P*-values of less than 0.05 were considered to indicate statistical significance and all *P*-values were based on two-sided tests. The analyses were performed with the SPSS statistical software package.

Results

Cross-sectional Analysis

Table 1 shows the baseline characteristics of the study subjects from the cohort of Leiden inhabitants aged 85 years and over (n = 666). A gender difference was observed with respect to smoking habits, alcohol consumption and the prevalence of hypertension and cancer. The MTHFR genotype distribution in the complete cohort was 46.5% (*ala*/*ala*), 44.4% (*ala*/*val*) and 9.0% (*val*/*val*). For the cross-sectional analysis, MTHFR genotype frequencies in the elderly subjects born in Leiden (56% of the complete cohort) were compared with those in young subjects aged 18–40 years whose families originated from the Leiden area (Table 2). Genotype frequencies in both groups were in agreement with the distribution predicted by the Hardy-Weinberg equilibrium. The prevalence of the *val*/*val* genotype in the control population (12.4%, n = 250) was consistent with the 10–18% reported for other populations greater than 200 subjects of European, North-American and Australian origin.[8–13,15–17]

The frequency of the *val* allele was significantly lower in elderly subjects than in young subjects (0.30 and 0.36, respectively; $\chi^2_{df=1}$ = 4.74, P = 0.030). This observation was illustrated by the over-representation of the *ala*/*ala* genotype (48.2% *vs* 40.0%) and the under-representation of *ala*/*val* (43.0% *vs* 47.6) and *val*/*val* genotype (8.8% *vs* 12.4%) in elderly subjects as compared with young subjects.

Since the mortality and specific death causes differ between men and women, and the exposure to factors which potentially modulate the effects of the MTHFR mutation, may vary between men and women, the association was explored for men and women separately. The prevalence of the *val*/*val* genotype in elderly men was significantly lower than in elderly women (4.4% and 10.8%, respectively; $\chi^2_{df=1}$ = 3.97, P = 0.046), whereas the *val*/*val* frequency was virtually the same in young men and women (12.2% and 12.6%, respectively; $\chi^2_{df=1}$ = 0.01, P = 0.93). Hence, the frequency of the *ala*/*val* and *val*/*val* genotypes were significantly reduced in elderly men as compared with

Table 1 Baseline characteristics of the 666 study subjects aged 85 years and over

Characteristics	*All subjects*	*Men*	*Women*	*Test for gender difference*
Number	666	188	478	
Born in Leiden – number (%)	365 (56%)	114 (61%)	251 (53%)	
Age – median (range)	89 (85–100)	89 (85–100)	89 (85–100)	*P*=0.48
History of myocardial infarction – %	7.8	8.7	7.5	*P*=0.57
History of cerebrovascular diseases – %	2.4	2.2	2.5	*P*=0.75
Hypertension[a] – %	22.6	8.7	28.0	*P*<0.0001
Diabetes – %	11.8	9.1	12.9	*P*=0.13
Cancer – %	7.1	10.5	5.8	*P*=0.014
Smoking – %	17.3	51.4	4.1	*P*<0.0001
Use of alcohol – %	25.3	49.4	16.1	*p*<0.0001

[a]Includes a self-reported history of hypertension, diastolic blood pressure >95 mmHg and/or the use of anti-hypertensive medication.

Table 2 MTHFR genotype distributions and estimated mortality risks in subjects aged 85 years and over and young subjects whose families originated from the same geographical region

MTHFR	*Subjects*		*Mortality*	*Test for trend*
Genotype	*Elderly* (%)	*Young*[a] (%)	*Risk (95% CI)*[b]	
all subjects				
ala/ala	176 (48.2%)	100 (40.0%)	1	
ala/val	157 (43.0%)	119 (47.6%)	1.3 (0.9–1.9)	
val/val	32 (8.8%)	31 (12.4%)	1.7 (1.0–3.0)	*P*=0.028
men only				
ala/ala	55 (48.2%)	50 (36.0%)	1	
ala/val	54 (47.4%)	72 (51.8%)	1.5 (0.9–2.5)	
val/val	5 (4.4%)	17 (12.2%)	3.7 (1.3–10.9)	*P*=0.011
women only				
ala/ala	121 (48.2%)	50 (45.0%)	1	
ala/val	103 (41.0%)	47 (42.3%)	1.1 (0.7–1.8)	
val/val	27 (10.8%)	14 (12.6%)	1.3 (0.6–2.6)	*P*=0.51

[a]Median age: 31 years (range 18–40); [b]Mortality risks and 95% CIs were estimated with the exposure odds ratio.

young men ($\chi^2_{df=1}$ = 6.40; *P* for trend = 0.011), but similar in elderly and young women ($\chi^2_{df=1}$ = 0.42; *P* for trend = 0.51) (Table 2).

Mortality risks were estimated on the basis of the MTHFR genotype distributions in elderly and young subjects. The mortality risks associated with the *ala*/*val* and the *val*/*val* genotype were estimated at 1.3-fold (95% CI, 0.9–1.9) and 1.7-fold (95% CI, 1.0–3.0) increases, respectively (Table 2). The mortality risk for men carrying the *val*/*val* genotype compared with those carrying the *ala*/*ala* genotype was estimated at 3.7 (95% CI, 1.3–10.9), whereas an increased mortality risk was virtually absent in women.

The elderly subjects were the survivors of a cohort born in Leiden between 1887 and 1901. Therefore an investigation was made of whether the young control population was likely to represent the Leiden genotype distribution of two generations earlier. The MTHFR genotype distribution in control subjects was independent of the number of grandparents born in Leiden (Table 3). This indicates that the selection criterion for the control population (i.e. either two Leiden-born parents or one Leiden-born parent and the other born within 12 km of Leiden) had been strict enough to obtain a population representing past Leiden genotype frequencies.

Table 3 MTHFR genotype distribution in young subjects dependent on their number of grandparents born in Leiden

MTHFR genotype	*Number of grandparents born in Leiden*			
	1 or more (*n*=203)	*2 or more* (*n*=178)	*3 or more* (*n*=120)	*4* (*n*=76)
ala/ala	38.4%	39.3%	44.2%	40.8%
a/a/val	49.8%	48.3%	43.3%	47.4%
val/val	11.8%	12.4%	12.5%	11.8%

Prospective Follow-up Study

During the 10-year follow-up period, 591 (89%) deaths occurred in the complete 85-plus cohort investigated in this study (*n* = 666; two subjects were lost to follow up). The cumulative survival of men and women stratified according to MTHFR genotype is shown in Figure 1. Men carrying the *val*/*val* genotype survived for a shorter time (*P* log-rank = 0.020). The median survival time of this group was 11 months compared with 38 months and 36 months for men with *ala*/*ala* and *ala*/*val* genotype, respectively. Among women, the MTHFR mutation was not associated with a difference in life expectancy (*P* log-rank = 0.16).

Overall, the *ala*/*val* and *val*/*val* genotypes were not associated with an increased mortality risk (gender and age-adjusted relative risk, 0.9 [95% CI, 0.7–1.0] and 0.9 [95% CI, 0.6–1.2], respectively). However, men homozygous for the mutation, but not heterozygous men, had a significantly increased mortality risk compared with men carrying the *ala*/*ala* genotype (age-adjusted relative risk, 2.0 [95% CI, 1.1–3.9]) (Table 4). This mortality risk did not appreciably change when adjusted for smoking, alcohol consumption, hypertension and diabetes (relative risk, 2.0 [95% CI, 0.9–4.4]). Among women, the MTHFR mutation was associated with marginally lower mortality risks, which bordered on the significant.

In the elderly cohort, the MTHFR mutation was not associated with a self-reported history of myocardial infarction or cerebrovascular disease (data not shown).

The mutation was also not associated with cardiovascular mortality either in men or women (Table 4). The mortality risk from cancer, however, was significantly higher among men with the *val*/*val* genotype (age-adjusted relative risk, 4.2 [95% CI, 1.3–13.5]), whereas among women carrying the *val*/*val* genotype the risk was not significantly different.

Discussion

In this study we have explored the possible detrimental effects of a disturbed methionine/homocysteine metabolism. This was done by investigating the association of the common MTHFR *ala*-to-*val* mutation with mortality in a cohort of men and women born between 1887–1901. It can be assumed that carriers of the MTHFR mutation, in general, have a mildly disturbed methionine/homocysteine metabolism during their whole life. The effect of the MTHFR mutation on mortality before the age of 85 was studied in a cross-sectional design that accounted for possible geographical differences in the MTHFR genotype distribution. The MTHFR mutation was associated with an increased mortality risk as indicated by an under-representation of the mutation in elderly compared with young subjects. The association was predominantly present in men. Men homozygous for the mutation had about a 4-fold increased mortality risk. Our findings are supported by the reduction of the *val*/*val* genotype

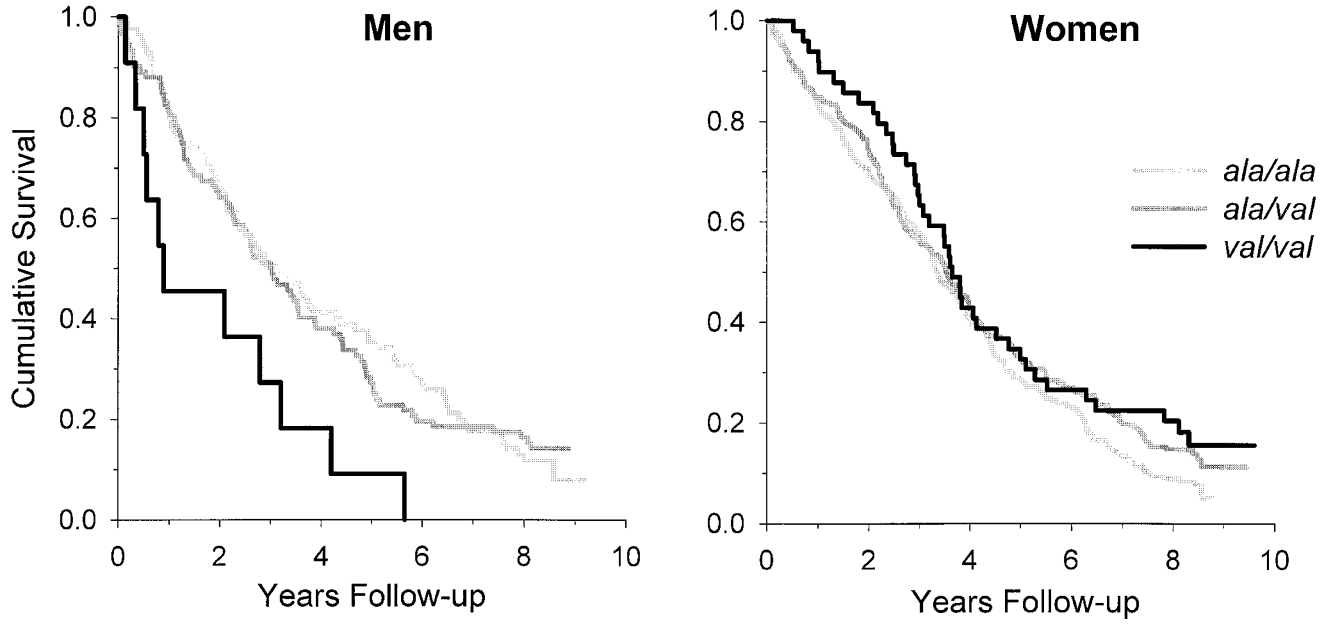

Figure 1 *Kaplan-Meier estimate of 10-year cumulative survival according to MTHFR genotype for men and women aged 85 years and over.*

Table 4 All-cause and cause-specific 10-year mortality risks according to MTHFR genotype in subjects aged 85 years and over

MTHFR genotype	*Number of subjects*	*All causes*		*Cardiovascular disease*		*Cancer*		*Infectious disease*	
		RR	(95% Cl)[a]	RR	(95% Cl)	RR	(95% Cl)	RR	(95% Cl)
men									
ala/*ala*	85	1		1		1		1	
ala/*val*	92	1.0	(0.7–1.4)	0.7	(0.4–1.2)	1.2	(0.6–2.6)	1.1	(0.4–3.1)
val/*val*	11	2.0	(1.1–3.9)	0.7	(0.2–3.1)	4.2	(1.3–13.5)	1.4	(0.1–14.6)
women									
ala/*ala*	223	1		1		1		1	
ala/*val*	204	0.8	(0.7–1.0)	0.9	(0.7–1.3)	0.9	(0.6–1.5)	0.6	(0.3–1.2)
val/*val*	49	0.7	(0.5–1.0)	0.9	(0.5–1.5)	0.5	(0.2–1.4)	0.3	(0.1–1.3)

[a]RR indicates the mortality risk as estimated with a Cox proportional hazard model adjusted for age at baseline.

observed in French centenarians[17] and the gradual decline in prevalence of the *val*/*val* genotype with increasing age found in Japanese subjects,[36] whereas two other cross-sectional studies[37,38] did not observe a decreased prevalence of the MTHFR mutation in old age. The design of these studies, however, did not extensively check for geographical variations in genotype distribution or gene-pool effects. Also, population differences in factors modulating the effect of the MTHFR mutation may have contributed to these variable results.

Mortality after the age of 85 years was examined during a 10-year follow-up period. The mutation conferred a 2-fold increased mortality risk in elderly men homozygous for the mutation, but not in women. The prospective follow-up study thus confirms the increased mortality risk associated with the MTHFR mutation observed in our cross-sectional analysis.

The relation of the MTHFR mutation with risk of cardiovascular disease is controversial. Some studies reported an association of the *val*/*val* genotype with an increased risk of cardiovascular disease,[4,14,18,23] whereas in other studies evidence for this relation was absent.[10,15,16,19,21,24–28] Here, we show that the MTHFR mutation was associated with an increased mortality in men before and after the age of 85 years but, by design, no data were available concerning the causes of death in middle age. Over the age of 85 years, cardiovascular diseases did not contribute to the increased mortality of men with the *val*/*val* genotype. Although half the elderly men and women died from cardiovascular disease (data not shown), the mutation was not associated with increased cardiovascular disease mortality. Two interpretations are compatible with these findings. The MTHFR mutation is not related to mortality due to cardiovascular diseases in middle and old age. Alternatively, carriers of the mutation are subject to an increased cardiovascular disease mortality before the age of 85 years, which has led to the selective survival of carriers who are less susceptible to cardiovascular diseases.

Although the numbers were small, we found that the increased mortality risk of men aged 85 years and over with the *val*/*val* genotype was largely explained by an increased risk of death due to cancer. An association between the MTHFR mutation and the risk of colorectal cancer was reported in two other prospective studies. United States health professionals[12] and physicians[29] carrying the *val*/*val* genotype were found to have a reduced risk of colorectal cancer. This protective effect was abolished by moderate alcohol consumption, probably because alcohol depletes folate.[39] It is not clear how the opposite effects of the MTHFR mutation on cancer risk in our population-based study and the previous studies can be explained. The MTHFR mutation may increase the risk of cancer especially in groups with a low folate intake, such as the elderly, rather than in well-nourished health professionals and physicians.[12,29]

From the present data it remains unclear why men carrying the MTHFR mutation were at an increased risk of mortality, whereas mortality in women carrying the mutation was not affected. Hormonal differences with respect to oestrogens are a less likely explanation for the association observed, since the gender-dependent association with mortality persisted after the age of 85 years when women are well beyond the menopause. Influence of oestrogens on the methionine/homocysteine metabolism is further refuted by the absence of a long-term effect of hormone replacement therapy on the level of plasma homocysteine.[40] Since the level of plasma folate is a critical modulator of the MTHFR mutation, differences in the level of plasma folate between men and women may also have contributed to the gender-dependent association. A previous study in elderly subjects, however, reported no difference in the level of plasma folate between men and women despite the higher folate intake of women.[41]

A clear gender difference was present with respect to smoking habits and alcohol consumption; 51% of the men and only 4% of the women reported smoking, and 49% of the men reported alcohol consumption *vs* 16% of the women. Smoking is associated with elevated plasma homocysteine,[15,42] whereas alcohol is a methyl group antagonist and depletes folate.[39] It may be hypothesised that the combined effects of smoking, alcohol consumption and the MTHFR mutation on the methionine/homocysteine metabolism might have led to an increased mortality risk in men but not in women. Especially among individuals from the 1887–1901 birth cohort, who were middle aged during the 1930s to 1960s; the majority of men smoked, whereas among women smoking was uncommon.

In conclusion, our data suggest that homozygosity for the MTHFR *ala*-to-*val* mutation increases the mortality risk in men both in middle and old age. Our study does not reveal the causes of death contributing to the increased mortality risk in middle age, but suggests that cancer rather than cardiovascular disease may be the primary cause of death in elderly men carrying the *val*/

val genotype. Larger prospective population-based studies are needed to confirm the effect of the MTHFR mutation on all-cause and cancer mortality. Interventions starting at a young age to restore a balanced methionine/homocysteine metabolism may prove to be beneficial to carriers of the *val*/*val* genotype.

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