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ORIGINAL PAPER

Folic acid supplementation, *MTHFR* and *MTRR* polymorphisms, and the risk of childhood leukemia: the ESCALE study (SFCE)

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Abstract
Purpose Fetal folate deficiency may increase the risk of subsequent childhood acute leukemia (AL), since folates are required for DNA methylation, synthesis, and repair, but the literature remains scarce. This study tested the hypothesis that maternal folic acid supplementation before or during pregnancy reduces AL risk, accounting for the SNPs rs1801133 (C677T) and rs1801131 (A1298C) in *MTHFR* and rs1801394 (A66G) and rs1532268 (C524T) in *MTRR*, assumed to modify folate metabolism.
Methods The nationwide registry-based case–control study, ESCALE, carried out in 2003–2004, included 764 AL cases and 1,681 controls frequency matched with the cases on age and gender. Information on folic acid supplementation was obtained by standardized telephone interview. The genotypes were obtained using high-throughput platforms and imputation for untyped polymorphisms. Odds ratios (OR) were estimated using unconditional regression models adjusted for potential confounders.
Results AL was significantly inversely associated with maternal folic acid supplementation before and during pregnancy (OR = 0.4; 95 % confidence interval: [0.3–0.6]). *MTHFR* and *MTRR* genetic polymorphisms were not associated with AL. However, AL was positively associated with homozygosity for any of the *MTHFR* polymorphisms and carriership of both *MTRR* variant

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alleles (OR = 1.6 [0.9–3.1]). No interaction was observed between *MTHFR*, *MTRR*, and maternal folate supplementation.

Conclusion The study findings support the hypothesis that maternal folic acid supplementation may reduce the risk of childhood AL. The findings also suggest that the genotype homozygous for any of the *MTHFR* variants and carrying both *MTRR* variants could be a risk factor for AL.

Keywords Childhood leukemia · Folic acid · Metabolism · MTHFR · MTRR · Gene–environment interaction

Introduction

Leukemia is the most common childhood cancer with about 470 new cases diagnosed each year in France [1, 2]. A few cases are explained by high doses of ionizing radiation, Down syndrome, a few rare genetic disorders, and certain chemotherapies, but the etiology of most cases of childhood leukemia remains largely unknown [3–5].

Several studies, 5 case–control studies [6, 9–12] and one incidence study [7, 8], investigated the hypothesis that maternal folic acid supplementation before or during pregnancy reduces the risk of childhood acute leukemia (AL), since folates are required for DNA methylation, synthesis, and repair. In addition, many studies [13–33] have attempted to correlate genetic polymorphisms of the folate metabolism pathway, mostly *MTHFR* polymorphisms, with susceptibility to childhood AL. They yielded inconsistent results. Most of them did not take maternal folic acid supplementation levels or folate status into account and did not investigate gene–environment interactions.

MTHFR activity is reduced in carriers of *MTHFR* C677T variant polymorphisms, and the reduction is greater among homozygotes (60–70 %) than among heterozygotes (35–40 %) [34].

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The A1298C variant of *MTHFR* also reduces *MTHFR* activity, albeit less markedly than the C677T variant [35]. Individuals carrying the A1298C variant allele do not appear to have higher serum homocysteine levels than homozygotes for the common allele A [36], but another study has suggested that heterozygosity for both variants A1298C and C677T has the same biochemical profile as homozygosity for the C677T variant [35].

The *MTRR* polymorphisms have been less studied. The *MTRR* A66G polymorphism decreases enzyme affinity for methionine synthase MTR [37].

The association between maternal folic acid supplementation before and during pregnancy and the risk of childhood AL in the national population-based case–control study, ESCALE, was analyzed. The roles of *MTHFR* and *MTRR* common polymorphisms, which are assumed to modify folate metabolism, were investigated.

Materials and methods

The ESCALE study was a national study conducted in 2003 and 2004 in mainland France (11 million children aged less than 15 years old) to investigate the role of infectious, environmental, and genetic factors in four childhood neoplastic diseases (AL, lymphoma, neuroblastoma, and brain tumor) [38–42]. This paper focuses on AL.

Case and control ascertainment

Cases

The cases were identified directly by the investigators of the French National Registry of Childhood Hematopoietic Malignancies (NRCH) [1] assigned to each pediatric oncology hospital department (Sect. Appendix). Eligible cases were children first diagnosed with one of the cancers under study between 1 January 2003 and 31 December 2004, aged less than 15 years, and resident in mainland France at the time of diagnosis. Cases who had been adopted, or whose biological mother could not be interviewed because she had died (10 cases), did not speak French (29 cases), or presented with a serious psychiatric disorder (15 cases), were not eligible. For ethical reasons, the mothers of children who had died (34 cases) or who were receiving hospital palliative care (7 cases) were not contacted. Of the 937 cases of AL identified during the study period, 843 cases were eligible. Out of the 843 eligible AL cases, 764 cases, consisting in 648 cases of acute lymphoblastic leukemia (ALL), 101 cases of acute myeloblastic leukemia (AML), and 15 cases of undifferentiated or biphenotypic AL, consented to participate (91 %). The cases were confirmed, documented, and classified by

leukemia cytological and immunological subtype by the NRCH.

Controls

The controls were selected from the French population in the years 2003 and 2004 using a quota-sampling method. A base of 60,000 phone numbers was randomly extracted from the national telephone directory. The set was representative of the population in terms of the administrative regions and urbanization. By incrementing each number by 1, a new set of 60,000 numbers was generated. The new set included unlisted numbers and had geographic and demographic distributions similar to those of the initial set (same first six digits, which determine the location of the line). Quotas were applied to make the age and gender distribution of the controls similar to that of all the ESCALE cases, based on estimates from the NRCH [1] and the Regional Childhood Cancer Registries [43], with age group: 0–1, 2, 3, 4, 5–6, 7–8, 9–11, and 12–14 years. The number of children <15 years of age in the household was forced to reflect that of the population by using quotas to prevent bias in the distribution of birth order, which may occur if the probability of a control being selected among phone subscribers depends on the size of the sibship. The expected number of children <15 years of age living in the household for a given age was obtained from the 1999 population census [INSEE (National Institute for Statistics and Economic Studies) 1999]. Thus, there were 48 quota strata: age (8 strata), gender (2 strata), and the number of children per household (1, 2, 3, or more). The controls were children free from cancer, who had not been adopted, whose biological mother was alive, who were free from serious psychiatric disorders, and who were French speaking. Out of the 50,217 phone numbers dialed, 22,584 did not connect to a household, 24,411 connected to ineligible households, and 862 to respondents who hung up before eligibility could be checked. The 2,361 remaining numbers were considered to be those of eligible households, 679 of which refused to participate. Thus, 1,681 mothers were interviewed (71.2 %).

Data collection

Using structured questionnaires, the same trained interviewers carried out the telephone interviews of the biological mothers of the cases and controls. Half of the cases' mothers were interviewed <4 months after the diagnosis (range: 1–24 months). The telephone questionnaire elicited information on demographic and socioeconomic characteristics, childhood medical history, childhood environment and lifestyle, and birth characteristics. The interviews also elicited parental occupational history, maternal exposure, and familial history of cancer, allergy and autoimmune disease, as well as the grandparent's countries of birth. The mothers were also asked to indicate whether they had taken folic acid or multivitamin supplements in the month preceding conception and in the first, second, and last quarter of the pregnancy. "Maternal folic acid supplementation" refers to maternal courses of supplements containing folic acid at a minimum dosage of 0.4 mg/day, while "multivitamin supplementation" refers to vitamin supplementation potentially containing folic acid at any dosage. The professional category of the parents was the higher of the maternal and paternal occupations at interview and was coded using the two-digit ILO classification (International Labor Organization, 1988). This variable was used as an indicator of socioeconomic status (SES).

With the parents' consent, blood samples were obtained from 729 (95.4 %) of the AL cases. Genome-wide genotyping was carried out on an Illumina 370 K Quad platform for the 588 AL cases from whom sufficient DNA had been obtained. The 6 cases with Down's syndrome and the 47 cases who did not comply with the quality control requirements were excluded from the analyses. The remaining 535 cases were successfully typed for 339,258 SNPs, with individual call rates of at least 97 %.

Saliva samples were obtained from 810 (48.2 %) of the controls, with parental consent, but 240 of the samples did not contain quantitatively or qualitatively sufficient DNA. The 570 remaining controls were genotyped for 4,551 selected SNPs using Infinium iSelect custom BeadChips, in compliance with the Illumina Infinium protocol. The individual call rate was greater than 95 % for 461 of the controls (80.9 %).

The comparability of the genotyping methods used for the cases and controls was checked by re-genotyping 96 cases on the platform used for the controls. The genotypes obtained by the 2 platforms were totally concordant.

Four polymorphisms were considered candidates in the present study because they were located within the genes *MTHFR* [rs1801131 (A1298C) and rs1801133 (C677T)] and *MTRR* [rs1801394 (A66G) and rs1532268 (C524T)] involved in folate metabolism. The SNPS rs1801133 and rs1532268 were genotyped for both the cases and controls, while rs1801131 and rs1801394 were only genotyped for the controls. The latter 2 SNPs were imputed for the cases using IMPUTE Version 2 software [44] from the SNPs located in the gene of interest or on either side of it (±100,000 bp), provided that they did not deviate significantly from the Hardy–Weinberg equilibrium in the controls. The CEU individuals of the 1000 Genomes Project (low coverage database) and the HapMap Project (Phases I + II + III release #28 database) were taken as a reference. One hundred iterations were performed, the first 20 of which (burn-in) were dropped. Imputation quality was assessed using the IMPUTE criteria *Info*

and *Certainty* >0.9. The imputed genotypes were considered if they were inferred with a probability ≥0.90 or considered missing values otherwise. In order to account for the imputation uncertainty, the analyses were also performed using regression logistic models weighted by the post-imputation probability of each genotype.

In order to control for potential population stratification bias, the analyses were restricted to the 493 cases and 441 controls who had at least two European-born grandparents. This indicator has been shown to predict, with 98.2 % sensitivity and 94.3 % specificity, the Caucasian status of the cases as determined by principal component analysis on 96,609 SNPs by the CEPH.

The 493 cases included in this analysis consisted in 434 cases of ALL (365 common B-cell ALL, 19 mature B-cell ALL, 40 T-cell ALL, and 10 unspecified ALL (ICCC codes: I.10: 98263, 98353, 98363, 98373, 99483) and 59 cases of ANLL [51 AML (ICCC codes: I.b: 98403, 98613, 98663, 98673, 98713, 98723, 98733, 98743, 98913, 98953, 98963, 98973, 99103) and 8 undifferentiated or biphenotypic leukemia (ICCC codes: I.e.: 98053, 99303)].

Statistical analysis

The SNPs of interest were individually analyzed under codominant and dominant coding, and grouped into combined variables for *MTHFR* (no *MTHFR* variant SNP; at least one heterozygous variant and no homozygous variant; homozygosity for any of the *MTHFR* variants) and *MTRR* (no *MTRR* variant SNP; at least one variant in rs1801394 and none in rs1532268; at least one variant in rs1532268 and none in rs1801394; at least one variant in both SNPs). The *MTHFR* and *MTRR* statuses were also combined to yield a *MTHFR–MTRR* variable.

Odds ratios (OR) and their 95 % confidence intervals (95 % CI) were estimated using unconditional logistic regression models (all AL) or polychotomous logistic regression (AL types) including the stratification variable used for quota-sampling age × gender (eight age groups for each gender) and the SES variable. The stability of the results was tested after additional adjustments and/or stratum analysis for factors related to childhood leukemia in the literature or in the ESCALE study (birth order, birth weight and breastfeeding, indoor maternal use of pesticides). The variables were not retained in the models if they did not change the first decimal of the estimated odds ratio.

The SAS® software package (version 9.2, SAS Institute Inc., Cary, NC, USA) was used for all the analyses.

The research was conducted in accordance with the principles of the Declaration of Helsinki (World Medical Association, 2004) and complied with all applicable international regulatory requirements including submission to an ethics committee (DGS No. 2003/0259).

Results

Case and control comparability

The distribution of the controls by age and gender was similar to that of the whole ESCALE case group but the controls were significantly older than the AL cases with a mean age (±SD) of 5.2 ± 3.7 for the cases and 5.5 ± 4.3 years for the controls (supplemental material, Table 1). All the strata contained more than one control per case for adjustment, with the most controls per case in the youngest strata. The control mothers reported higher paternal education and more qualified parental professional status than the case mothers (Table 1).

Maternal supplementation during the index pregnancy and risk of acute leukemia

In the ESCALE study, 172 (10.7 %) of the control mothers and 32 (4.4 %) of the case mothers reported folic acid supplementation during the preconception period or index pregnancy. The mothers of 43 cases (5.6 %) and 70 controls (4.1 %) could not remember whether they took folic acid or not.

Childhood leukemia was negatively and significantly associated with folic acid supplementation (OR = 0.4, 95 % CI = [0.3–0.6]) but the association was not limited to supplementation initiated before or during the first trimester of pregnancy (Table 2). The relationships with ALL and ANLL seemed similar, but were based on small numbers for ANLL (ALL: OR = 0.4, 95 % CI = [0.3–0.6]; NALL: OR = 0.3, 95 % CI = [0.1–0.9]). The OR did not vary substantially across immunological or ploidy subtypes or according to TEL-AML1 or MLL status (Table 3). The association was also negatively significant when we considered the risk of childhood AL and the maternal folic acid or multivitamin supplementation (OR = 0.6, 95 % CI = [0.5–0.8]) (Table 2).

Some perinatal characteristics may introduce confounding in the relationship between maternal folic acid supplementation and AL. The controls whose mothers received folic acid supplementation had the same birth order and birth weight as the other controls, but they had been breastfed more often (64.5 % vs. 49.9 %) and presented with birth defects more often (4.7 % vs. 2.9 %). Neither adjustment for those factors in addition to the stratification variable age * gender and parental SES, nor exclusion of the cases with Down's syndrome changed the results.

Moreover, stratifying by period of birth did not show any heterogeneity of the negative association between AL and maternal folate supplementation over time (before 1996: OR = 0.4 [0.2–1.0]; 1996–2000: OR = 0.3

Table 1 Distribution of cases and controls by educational level, professional category, and area of residence

	Controls ($N = 1681$)		AL[a] ($N = 764$)		OR[b]	95 % CI
	N	(%)	*N*	(%)		
Maternal educational level						
Baccalaureate +2 years or advanced degree	701	42	299	39	1.0	Ref.
Baccalaureate	320	19	138	18	1.0	[0.8–1.3]
Vocational training (CAP, BEP)	500	30	247	32	1.2	[0.9–1.4]
None	159	9	80	10	1.2	[0.9–1.6]
Paternal educational level						
Baccalaureate +2 years or advanced degree	601	36	230	30	1.0	Ref.
Baccalaureate	236	14	114	15	1.3	[1.0–1.7]
Vocational training (CAP, BEP)	662	40	313	41	1.2	[1.0–1.5]
None	165	10	99	13	1.6	[1.2–2.2]
Parental professional category						
Managers, intellectual/intermediate professions	715	43	279	37	1.0	Ref.
Administrative and sales workers	477	28	224	29	1.2	[1.0–1.5]
Service workers	215	13	96	13	1.2	[0.9–1.5]
Factory/agricultural workers, unemployed	274	16	165	22	1.5	[1.2–1.9]
Area of residence at interview						
Rural	601	36	250	33	1.0	Ref.
Mixed	391	23	183	24	1.1	[0.9–1.4]
Urban	689	41	329	43	1.1	[0.9–1.4]

[a] *AL* Acute leukemia

[b] OR: (odds ratio) and 95 % confidence interval (95 % CI) estimated by unconditional logistic regression models including the stratification variable age × gender

[0.2–0.6]; 2001–2005: OR = 0.5 [0.3–0.9]). The results by socioeconomic category were also similar ("Managers, intellectual/intermediate professions": OR = 0.6 [0.4–1.0]; "Administrative and sales workers": OR = 0.1 [0.04–0.5]; "Service workers/Factory/agricultural workers, unemployed": OR = 0.3 [0.1–0.7]).

Selection of Caucasian children whose parents agreed for the child DNA sampling may have biased the results. The subsample used for the analysis of *MTHFR* and *MTRR* polymorphisms selected parents who were more educated and in more qualified socioeconomic categories, but the results for folate supplementation were the same as those for the entire sample.

The sensitivity analyses showed that the inverse relationship with folic acid supplementation remained when the controls with missing values were included in the group without supplementation and the cases with missing values were included in the supplemented group in a proportion such that maternal supplementation was 5 times as prevalent as that of the controls (OR 0.7 [0.5–0.9]).

Associations between MTHFR and MTRR polymorphisms and AL

In the control sample, there was no evidence of a deviation from the Hardy–Weinberg equilibrium for the candidate SNPs in MTHFR and MTRR.

MTHFR polymorphisms rs1801131 (A1298C) and rs1801133 (C677T) were not individually associated with ALL (Table 4). Homozygosity for any of the two variant alleles was associated with increased odds ratios (OR = 1.3 [0.8–2.4]) but the difference was not statistically significant. However, there was a positive relationship between homozygosity for the variant allele of rs1801133 and ANLL, and the relationship was on the borderline of significance. Interestingly, none of the homozygotes for the variant allele of one SNP carried a variant allele for the other SNP.

MTRR polymorphisms rs1801394 (A66G) and rs1532268 (C524T) were not significantly associated with AL taken as a whole, but the variant allele of rs1801394 tended to be inversely associated with ALL, although on the borderline of significance (Table 4). Being homozygous for one *MTHFR* polymorphism and carrying both *MTRR* variant alleles were positively associated with AL, ALL, and ANLL, albeit not significantly (OR = 1.6 [0.9–3.1] for AL) (Table 4). There were no interactions between maternal folic acid supplementation and any of the SNPs under study or with the combination of *MTHFR* and *MTRR* polymorphisms ($p = 0.90$). In a multivariate model, the association was stronger and of borderline significance after adjustment for maternal folic acid supplementation (OR = 1.8 [1.0–3.4] for AL) (Table 5).

Table 2 Relationship between childhood leukemia and periconception supplementation

	Any AL[c]				ALL[c]			ANLL[c]		
	Controls [$n = 1681$]	Cases [$n = 764$]	OR[a]	95 % CI	Cases ($n = 648$)	OR[b]	95 % CI	Cases ($n = 116$)	OR[b]	95 % CI
Folic acid supplementation										
No	1,439	689	1.0	Ref.	586	1.0	Ref.	103	1.0	Ref.
Yes	172	32	0.4	[0.3–0.6]	28	0.4	[0.3–0.6]	4	0.3	[0.1–0.9]
Missing	70	43	–		34	–		9	–	
First supplementation										
No folic acid supplementation	1,439	689	1.0	Ref.	584	1.0	Ref.	105	1.0	Ref.
Preconception or first trimester	70	10	0.3	[0.2–0.6]	9	0.3	[0.2–0.7]	1	0.2	[0.0–1.4]
Second trimester	56	15	0.6	[0.3–1.0]	13	0.6	[0.3–1.1]	2	0.5	[0.1–2.0]
Third trimester	37	4	0.3	[0.1–0.7]	3	0.2	[0.1–0.8]	1	0.4	[0.1–2.8]
Missing	79	46	–		37	–		9	–	
Folic acid or multivitamin supplementation										
No	1,350	643	1.0	Ref.	548	1.0	Ref.	95	1.0	Ref.
Yes	276	83	0.6	[0.5–0.8]	72	0.7	[0.5–0.9]	11	0.6	[0.3–1.1]
Missing	55	38	–		28	–		10	–	

[a] Odds ratio (OR) and 95 % confidence interval (95 % CI) estimated by unconditional logistic regression models including the stratification variable age × gender and socioeconomic status

[b] Odds ratio (OR) and 95 % confidence interval (95 % CI) estimated by polychotomous regression models including the stratification variable age × gender and socioeconomic status

[c] *AL* Acute leukemia, *ALL* acute lymphoblastic leukemia, *ANLL* acute non-lymphoblastic leukemia

Discussion

Overall, the results suggest that childhood AL is strongly negatively and significantly associated with maternal folic acid supplementation during the index pregnancy. The strengths of the association for ALL and ANLL were very similar. The association with maternal folic acid supplementation was stronger for mothers reporting having used supplements containing folic acid at the recommended dosage than for mothers reporting folic acid and/or multivitamin supplementation. Carrying both *MTRR* (rs1801394 and rs1532268) variant alleles while being homozygous for any variant allele of the *MTHFR* (rs1801133 and rs1801131) polymorphisms was positively associated with ALL and ANLL.

The cases were identified using the data collection system of the NRCH and the case mother participation rate was very high (91 %), which limited case selection bias. The main reason for non-inclusion was the child's poor state of health or death. Maternal folic acid supplementation is unlikely to be directly related to the severity of the disease or short-term survival. In addition, with regard to ESCALE, maternal supplementation was reported for none of the 15 children who died after their inclusion.

The quota-sampling process used for selecting population controls successfully ensured that the responding controls had the same distribution as the case group with regard to gender and age, and the same distribution as the overall population with regard to region and birth order: 41 and 37 % of the ESCALE controls born in 1995, or 2003 were first- and second-born children compared with 43 and 34 % in the French national perinatal surveys [45, 46]. With regard to parental education, 43 % of the children had graduate mothers, compared with 39 % in the perinatal surveys. Selection of more educated mothers is therefore possible, but limited.

The control parents appeared to belong to slightly higher socioeconomic categories than the case parents, which is likely to reflect slightly biased selection of the controls who agreed to participate in the study. Selection through acceptance of biological sample donation slightly increased the differential. Since parental SES was positively and significantly associated with folic acid supplementation among controls, under-representation of poorer households may have resulted in over-representation of maternal supplementation in the controls and overestimation of the relationship. However, the impact is unlikely to be marked since adjustments for (or stratification by) maternal,

Table 3 Relationship between childhood leukemia and periconception supplementation in immunological and ploidy subtypes and by TEL-AML1 or MLL status

	Folic acid		OR[a]	95 % CI
	Yes	No		
All ALL[b]	28	586	0.4	[0.3–0.6]
Immunophenotype				
Common/pre-B ALL	24	461	0.4	[0.3–0.7]
Burkitt ALL	1	29	_	
T-cell ALL	2	63	0.4	[0.1–1.5]
Ploidy				
Normal karyotype	12	161	0.6	[0.3–1.2]
Pseudodiploidy	6	171	0.3	[0.1–0.7]
Moderate hyperdiploidy	1	73	_	
Massive hyperdiploidy (>50 chromosomes)	9	157	0.5	[0.2–1.0]
TEL-AML1 status				
Negative	15	334	0.4	[0.2–0.7]
Positive	6	110	0.5	[0.2–1.1]
MLL status				
No	16	414	0.3	[0.2–0.6]
Yes	0	22	_	

[a] Odds ratio (OR) and 95 % confidence interval (95 %CI) estimated by polychotomous regression models including the stratification variable age × gender and socioeconomic status

[b] ALL: acute lymphoblastic leukemia

paternal education or parental SES did not change the results. Moreover, the associations were quite stable across strata of parental education and SES.

Data on maternal folic acid supplementation were collected retrospectively by interview, and misclassifications are therefore likely to have occurred. The use of a standardized questionnaire and the very similar conditions for the interviews of case and control mothers should have limited differential misclassifications, particularly since folic acid supplementation is not usually elicited by the physician at the time of diagnosis. In addition, the question of the relationship between maternal folic acid supplementation and childhood AL is not usually a public concern, while differential recall bias is more likely to occur for exposures assumed to be a risk for the disease or socially considered undesirable [47].

Maternal folic acid supplementation during the index pregnancy was less often reported in the present study (11 % for folic acid and 17 % for folic acid and multivitamins for the controls) than in most of the recent studies. Maternal supplementation may have been under-reported, particularly since it was elicited by interview some time after the pregnancy. The French campaign to promote folic acid supplementation was launched later, in December 2004, at the end of the ESCALE study. No folate fortification of the food supply is implemented in France. In a survey conducted in maternity wards in Paris area in 1999 [48], 24.3 % of the mothers had taken a supplement containing folic acid for 1 month before conception and for 2 months after the beginning of pregnancy. In the present study, maternal folic acid or multivitamin supplementation (including folic acid supplementation with less than the 400 mg/day currently recommended) was reported for 27 % of the controls born in the Paris area in 1997–2001.

Restricting the sample to subjects of European ancestry, based on the grandparents' country of birth, reduced the potential for population stratification bias. The distribution of the genotypes of the controls did not deviate from the Hardy–Weinberg equilibrium. The controls who were genotyped did not differ from the other controls in terms of maternal folic acid supplementation. Confounding by other factors related to AL in the previous analyses was accounted for by adjustment, exclusion, and stratum-specific analyses. The results were not substantially changed.

The cases and controls were genotyped separately using different platforms. However, the concordance between the two platforms was total for the SNPs they had in common. For the cases, the genotypes of two of the four candidate SNPs, rs1801131 in *MTHFR* and rs1801394 in *MTRR,* were imputed with the widely used IMPUTE program, which is known to be of a good efficacy and accuracy [49, 50]. The genotypes were imputed with an aposteriori probability greater than 0.90 and with good-quality criteria. The results remained stable when the aposteriori probabilities were considered in a weighted logistic regression analysis.

The published data on the impact of the two *MTHFR* polymorphisms on plasma homocysteine levels suggest they comply with a codominant model [36, 51]. They were therefore combined in that manner, assuming that the last category (homozygosity for any of the MTHFR variants) gives rise to the greatest decrease in MTHFR enzyme activity. It is noteworthy that all the individuals homozygous for the variant of one of the *MTHFR* polymorphisms were homozygous for the ancestral allele for the other polymorphism. Published data on the *MTRR* polymorphisms suggest that they lower enzyme affinity for methionine synthase [52], with no indication that they depart from codominant models. They were therefore combined with the assumption that carrying the 2 variants might have more impact on metabolism. For the combined variable, *MTHFR* and *MTRR* polymorphisms, the last category of the *MTHFR* combined variable was split by *MTRR* status, in order to distinguish the class with the highest expected impact on folate metabolism.

Three case–control studies have analyzed the association between ALL and folic acid supplementation, alone or in combination with other supplements, during the index

Table 4 Relationship between childhood leukemia and *MTHFR* and *MTRR* polymorphisms

	AL[c]				ALL[c]			ANLL[c]		
	Controls ($n = 441$)	Cases ($n = 493$)	OR[a]	95 % CI	Cases ($n = 434$)	OR[b]	95 % CI	Cases ($n = 59$)	OR[b]	95 % CI
MTHFR polymorphisms										
MTHFR rs1801133 (C677CT)										
CC	178	191	1.0	Ref.	172	1.0	Ref.	19	1.0	Ref.
CT	193	234	1.1	[0.8–1.4]	208	1.0	[0.8–1.4]	26	1.1	[0.6–2.2]
TT	56	68	1.1	[0.7–1.8]	54	1.0	[0.6–1.6]	14	2.2	[1.0–4.8]
CT + TT	249	302	1.1	[0.8–1.4]	262	1.0	[0.8–1.4]	40	1.4	[0.8–2.5]
Missing	14	0	–			–			–	
MTHFR rs1801131 (A1298C)[d]										
AA	208	212	1.0	Ref.	182	1.0	Ref.	30	1.0	Ref.
AC	188	200	0.9	[0.7–1.3]	179	1.0	[0.7–1.3]	21	0.7	[0.4–1.4]
CC	45	50	1.0	[0.6–1.7]	45	1.1	[0.7–1.8]	5	0.8	[0.3–2.1]
AC + CC	233	250	0.9	[0.7–1.3]	224	1.0	[0.7–1.4]	26	0.7	[0.4–1.3]
Missing	0	31	–		28	–		3	–	
MTHFR rs1801133 and rs1801131										
Both ancestral	48	39	1.0	Ref.	34	1.0	Ref.	5	1.0	Ref.
At least one variant but none homozygous	278	306	1.2	[0.7–2.0]	273	1.3	[0.8–2.2]	33	0.9	[0.3–2.6]
Homozygous for any of the *MTHFR* variants	101	117	1.4	[0.8–2.3]	99	1.3	[0.8–2.4]	19	1.5	[0.5–4.5]
Missing	14	31	–		28	–		3	–	
MTRR polymorphisms										
MTRR rs1801394 (A66G)[d]										
AA	95	122	1.0	Ref.	112	1.0	Ref.	10	1.0	Ref.
AG	226	211	0.7	[0.5–1.0]	187	0.7	[0.5–1.0]	24	1.0	[0.4–2.2]
GG	120	132	0.9	[0.6–1.3]	110	0.8	[0.5–1.2]	22	1.7	[0.8–4.0]
AG + GG	346	343	0.8	[0.6–1.1]	297	0.7	[0.5–1.0]	46	1.2	[0.6–2.6]
Missing	0	28	–		25	–		3	–	
MTRR rs1532268 (C524T)										
CC	181	180	1.0	Ref.	155	1.0	Ref.	25	1.0	Ref.
CT	192	236	1.3	[0.9–1.7]	209	1.3	[0.9–1.7]	27	1.1	[0.6–2.0]
TT	68	77	1.0	[0.7–1.6]	70	1.1	[0.7–1.7]	7	0.7	[0.3–1.8]
CT + TT	260	313	1.2	[0.9–1.6]	279	1.2	[0.9–1.6]	34	1.0	[0.6–1.8]
MTRR rs1801394 and rs1532268										
Both ancestral	44	52	1.0	Ref.	45	1.0	Ref.	7	1.0	Ref.
rs1801394 variant only	51	70	1.2	[0.7–2.2]	67	1.4	[0.7–2.5]	3	0.5	[0.1–2.0]
rs1532268 variant only	137	117	0.8	[0.5–1.3]	99	0.8	[0.4–1.3]	18	0.9	[0.4–2.5]
Both variants	209	226	0.9	[0.6–1.5]	198	0.9	[0.6–1.6]	28	0.9	[0.4–2.4]
Missing	0	28	–		25	–		3	–	
MTHFR and *MTRR* polymorphisms										
Both *MTHFR* SNPs ancestral	48	39	1.0	Ref.	34	1.0	Ref.	5	Ref.	
At least one *MTHFR* variant but none homozygous	278	306	1.2	[0.8–2.0]	273	1.3	[0.8–2.1]	33	1.0	[0.3–2.6]
Homozygous for any of the *MTHFR* variants										
No *MTRR* variant or only one *MTRR* variant	58	53	1.1	[0.6–2.0]	41	1.0	[0.5–1.8]	12	1.6	[0.5–5.1]

Table 4 continued

	AL[c]				ALL[c]			ANLL[c]		
	Controls ($n = 441$)	Cases ($n = 493$)	OR[a]	95 % CI	Cases ($n = 434$)	OR[b]	95 % CI	Cases ($n = 59$)	OR[b]	95 % CI
Both *MTRR* variants	43	57	1.6	[0.9–3.1]	50	1.7	[0.9–3.2]	7	1.5	[0.4–5.1]

[a] Odds ratio (OR) and 95 % confidence interval (95 % CI) estimated by unconditional logistic regression models including the stratification variable age × gender and socioeconomic status

[b] Odds ratio (OR) and 95 % confidence interval (95 % CI) estimated by polychotomous regression models including the stratification variable age × gender and socioeconomic status

[c] *AL* acute leukemia, *ALL* acute lymphoblastic leukemia, *ANLL* acute non-lymphoblastic leukemia

[d] SNPs imputed for cases and genotyped for controls

pregnancy. An Australian study showed a strong and significant inverse association of the same order of magnitude as that reported herein (OR = 0.40 [0.21–0.73]) [12], but the two subsequent studies in New Zealand [6] and Australia [9] reported a null association. Two other case–control studies considered folic acid supplementation, but did not distinguish between folic acid and other vitamins. Supplementation with vitamins including folic acid was not associated with ALL in a Canadian case–control study [11], but a German study reported an inverse relationship with vitamins, folic acid, and/or iron supplementation [10]. A study in Ontario conducted to evaluate changes in ALL incidence found no change in ALL incidence after a folic acid food fortification program, first in infant leukemia [7] and later in older children [8].

Several studies investigated the relationship between AL and *MTHFR* polymorphisms C677T or A1298C [13–17, 19–22, 24–33]. Published meta-analyses reported inverse or null association between ALL and *MTHFR* C677T [53–57], and no association with *MTHFR* A1298C polymorphism.

MTRR polymorphisms have been less investigated. The only study on C524T found no association with ALL [18]. The A66G polymorphism was not associated with ALL in one study [31] and inversely related to ALL in three studies, significantly [18] or not [17, 23]. No association was found in another study [25]. Most of the published papers on *MTHFR* C677T and A1298C polymorphisms compared case series with unmatched control series. Potential confounders were only considered in a few studies. In the present study where controls were carefully selected from the population which has given rise to cases, and in which potential confounders were taken into account, no association was observed for any of the four SNPs.

Few studies investigated combinations of polymorphisms. The study of Petra et al. [25] showed a non-significant inverse association with both homozygosity for the *MTHFR* C677T variant allele (*T*) and carriership of the *MTRR* A66G variant allele (*G*). Interestingly, a recent publication on the Californian NCCLS study [23] reported on a comprehensive ensemble of polymorphisms in genes involved in the folate pathway and suggested a positive association between ALL and a specific 3-SNPs haplotype block in *MTRR* among Hispanics.

The variants analyzed herein are not included in that block. However, in this Californian study, the OR of association between rs1801394 and AL was 0.9 [0.6–1.4], close to the result reported herein. The present study is the first to consider together the SNPs rs1801133 (C677T) and rs1801131 (A1298C) in *MTHFR* and rs1801394 (A66G) and rs1532268 (C524T) in *MTRR,* which are assumed to modify the activity of the enzymes.

Folic acid supplementation may influence blood homocysteine levels, as suggested in a study [58] in which homozygotes for the *MTHFR* C677T variant allele tended to have normal blood homocysteine levels when folate intake was adequate. In that case, the decreased activity of *MTHFR* may not lead to DNA hypomethylation [51] or misincorporation of uracil in DNA [59]. Thus, the C677T polymorphism may reduce cancer risk if folate intake is adequate or, on the contrary, increase cancer risk if folate intake is inadequate [60]. In the present study, *MTHFR* and *MTRR* polymorphisms and their combinations did not interact with the relationship between AL and maternal folic acid supplementation. A case-only analysis [61] observed also no interaction between the *MTHFR* genotype and folate supplementation in association with ALL.

The Californian study [23] found an interaction between some *MTRR* polymorphisms and maternal dietary and supplemental folate intake, since the *MTRR* SNPs were positively associated with ALL in the group with the lowest maternal intake and inversely associated with ALL in the other group. There was no interaction between folate intake and any of the *MTHFR* polymorphisms or the *MTRR* A66G polymorphism. Two studies stratified the analyses of *MTHFR* polymorphisms before and after a folate supplementation recommendation campaign and found no association for the German study [28] and a negative association restricted to the period preceding the campaign for the Canadian study [21].

Table 5 Relationship between childhood leukemia and combinations of *MTHFR* and *MTRR* polymorphisms with maternal folate supplementation

	Controls ($n = 404$)	AL[c]			ALL[c]			ANLL[c]		
		Cases ($n = 430$)	OR[a]	95 % CI	Cases ($n = 434$)	OR[b]	95 % CI	Cases ($n = 59$)	OR[b]	95 % CI
Multivariate analysis										
Folic acid supplementation										
No folic acid supplementation	360	421	1.0	Ref.	368	1.0	Ref.	53	1.0	Ref.
Folic acid supplementation	44	17	0.3	[0.2–0.6]	16	0.3	[0.2–0.6]	1	0.2	[0.0–1.0]
MTHFR/*MTRR* status										
Both *MTHFR* SNPs ancestral	48	39	1.0	Ref.	34	1.0	Ref.	5	1.0	Ref.
At least one *MTHFR* variant but none homozygous	278	306	1.3	[0.8–2.1]	273	1.4	[0.8–2.3]	33	1.0	[0.6–2.8]
Homozygous for any of the *MTHFR* variants										
No *MTRR* variant or only one *MTRR* variant	58	53	1.2	[0.6–2.2]	41	1.0	[0.5–2.0]	12	1.9	[0.6–6.0]
Both *MTRR* variants	43	57	1.8	[1.0–3.4]	50	1.8	[0.9–3.5]	7	1.7	[0.5–6.0]

[a] Odds ratio (OR) and 95 % confidence interval (95 % CI) estimated by unconditional logistic regression models including the stratification variable age × gender and socioeconomic status

[b] Odds ratio (OR) and 95 % confidence interval (95 % CI) estimated by polychotomous regression models including the stratification variable age × gender and socioeconomic status

[c] *AL* acute leukemia, *ALL* acute lymphoblastic leukemia, *ANLL* acute non-lymphoblastic leukemia

In conclusion, the results reported herein support the hypothesis that maternal folic acid supplementation before or during pregnancy may reduce the risk of AL. They also suggest that the genotype homozygous for any of the *MTHFR* variants and carrying both *MTRR* variants may be a risk factor for AL.

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Conflict of interest No potential conflicts of interest were disclosed.

Appendix

See Table 6.

Table 6 SFCE investigators of the ESCALE study

Principal investigator	Hospital	City (France)
André Baruchel	Hôpital Saint-Louis/Hôpital Robert Debré	Paris
Claire Berger	Centre Hospitalier Universitaire	Saint-Etienne
Christophe Bergeron	Centre Léon Bérard	Lyon
Jean-Louis Bernard	Hôpital La Timone	Marseille
Yves Bertrand	Hôpital Debrousse	Lyon
Pierre Bordigoni	Centre Hospitalier Universitaire	Nancy
Patrick Boutard	Centre Hospitalier Régional Universitaire	Caen
Gérard Couillault	Hôpital d'Enfants	Dijon
Anne-Sophie Defachelles	Centre Oscar Lambret	Lille
François Demeocq	Hôpital Hôtel-Dieu	Clermont-Ferrand
Stéphane Ducassou	Hôpital Pellegrin Tripode	Bordeaux
Alain Fischer	Hôpital des Enfants Malades	Paris
Virginie Gandemer	Centre Hospitalier Universitaire—Hôpital Sud	Rennes
Stéphanie Haouy	Hôpital Arnaud de Villeneuve	Montpellier
Charlotte Jubert Hôpital Pellegrin Tripode	Bordeaux	
Jean-Pierre Lamagnere	Centre Gatien de Clocheville	Tours
Françoise Lapierre	Centre Hospitalier Universitaire Jean Bernard	Poitiers
Guy Leverger	Hôpital Armand-Trousseau	Paris
Patrick Lutz	Hôpital de Hautepierre	Strasbourg
Geneviève Margueritte	Hôpital Arnaud de Villeneuve	Montpellier
Françoise Mechinaud	Hôpital Mère et Enfants	Nantes
Gérard Michel	Hôpital La Timone	Marseille
Frédéric Millot	Centre Hospitalier Universitaire Jean Bernard	Poitiers
Martine Münzer	American Memorial Hospital	Reims
Brigitte Nelken	Université Lille Nord de France Lille	
Hélène Pacquement	Institut Curie	Paris
Brigitte Pautard	Centre Hospitalier Universitaire	Amiens
Alain Pierre-Kahn	Hôpital Enfants Malades	Paris
Christophe Piguet	Centre Hospitalier Régional Universitaire	Limoges
Geneviève Plat	Hôpital des Enfants	Toulouse
Emmanuel Plouvier	Centre Hospitalier Régional	Besançon
Xavier Rialland	Centre Hospitalier Universitaire	Angers
Hervé Rubie	Hôpital des Enfants	Toulouse
Christine Soler	Fondation Lenval	Nice
Dominique Valteau-Couanet	Institut Gustave Roussy	Villejuif
Jean-Pierre Vannier	Hôpital Charles Nicolle	Rouen

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