Maternal RFC1 Gene Polymorphisms and Neural Tube Defects: A Case–Control Study in Ethiopia

Hasset Tamirat Molla * , Dawd Gashu , Winyoo Chowanadisai , Barbara Stoecker

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Article

Maternal RFC1 Gene Polymorphisms and Neural Tube Defects: A Case–Control Study in Ethiopia

Hasset Tamirat Molla [1,*], Dawd Gashu [1], Winyoo Chowanadisai [2] and Barbara Stoecker [2]

[1] Center for Food Science and Nutrition, Addis Ababa University, Ethiopia
[2] Department of Nutritional Science, Oklahoma State University, Stillwater, OK, 74078, USA
* Correspondence: hasset.tamirat@aau.edu.et; Tel.: +251911681054

Abstract

Background: Etiologies of neural tube defects (NTDs) are multifactorial. Genetic, epigenetic and environmental factors may contribute to their reported variation in prevalence across the globe. Ethiopia has among the highest reported NTD prevalence globally, making investigation of genetic determinants in this high-risk population particularly important for advancing understanding of NTD etiology. Genes involved in folate metabolism, such as the reduced folate carrier 1 (RFC1), have been investigated for the potential associations with NTDs, but findings throughout the literature remain inconsistent and inconclusive. **Objective**: The aim of this study was to determine an association of RFC-1 polymorphism at rs1131596 and rs1051266 loci (functional variants previously implicated in folate transport efficiency and NTD susceptibility) among mothers with the occurrence of NTDs in their offspring in Ethiopia. **Methods**: A case-control study involving 250 mothers (187 controls and 63 cases) of children with or without NTDs was conducted in Addis Ababa, Ethiopia between April, 2022, to September, 2024. A total of 250 maternal whole blood samples were systematically collected and subjected to genetic analysis at loci rs1131596 and rs1051266 by PCR (polymerase chain reaction) and Sanger sequencing. **Results**: Detection of heterozygous (TC) and homozygous (CC) genotypes for SNP rs1131596 (-43T>C) in the RFC1 gene was 27.2%, with heterozygous (TC) comprising 10.4% and homozygous (CC) 16.8 %. In contrast, for the rs1051266 (80A>G), the prevalence of the AG polymorphism was 28% while the GG polymorphism was 16.4%, resulting in a cumulative prevalence of 44.4%. The presence of maternal RFC-1 polymorphism at these two locations did not show significant association ($p = 0.601$ & $p = 0.225$ respectively) with increased risk for NTD births. **Conclusion**: This study did not reveal significant association between maternal RFC-1 gene polymorphisms and NTD-affected births. Comprehensive whole-genome sequencing of affected off springs is essential to identify specific mutations or polymorphisms that may individually or collaboratively affect the risk of NTDs in the Ethiopian context.

Keywords: single nucleotide polymorphism (SNP); neural tube defects (NTD); RFC1 gene

1. Introduction

Neural tube defects (NTDs) are structural defects of the central nervous system that affect the brain and spinal column during the first month after conception [1]. NTDs occur due to the partial or complete failure of neural tube closure [2,3]. Ranging from mild to severe symptoms, the three main defects (anencephaly, encephalocele, and spina bifida) pose significant challenges. While anencephaly is typically associated with early mortality, individuals with spina bifida can survive with appropriate medical care, even though they often face substantial disability and limited life expectancy [4]. NTDs rank as the second most prevalent birth defect following orofacial defects [5].

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The global prevalence of neural tube defects is estimated at 18.6/10,000 (uncertainty interval: 15.3–23.0) live births, where approximately 75% of cases result in under-five mortality [6]. An estimate of two cases per 1000 births would annually approximate 214,000–322,000 affected pregnancies worldwide [5,7].

Most of the mortality burden of NTDs is in low- and middle-income countries like Ethiopia. NTD occurs at a markedly higher rate in North Ethiopia compared to the global norm, with an estimated 131 affected infants per 10,000 births, in contrast to the worldwide average of 18.6 per 10,000 [8]. A recent study underscores the severity of the issue in Ethiopia, revealing the country's high adjusted mortality fraction (7.5%) and adjusted mortality rate (104.0 per 10,000 births) attributed to NTDs if compared to South-East Asia (13.1 per 10,000 births) and other Sub-Saharan countries (14.2 per 10,000 births)[9]. Despite these alarming statistics, there is a significant gap in our understanding of the potential genetic risk factors associated with the occurrence of births affected with NTDs.

The etiology of NTDs is multifactorial, involving an interplay of genetic, epigenetic, and environmental determinants. Genetic contributions are estimated to account for up to 70% of the observed variance in NTD prevalence, highlighting their predominant role in disease susceptibility [10]. Among the genetic factors, genes involved in folate metabolism namely reduced folate carrier (RFC1) and Methyl TetrahydroFolate Reductase (MTHFR) have been primarily indicated to have association with births affected with NTDs with varying degrees of supportive evidence [11–13].

Case-control studies have found no significant association between the common RFC1 A80G (rs1051266) polymorphism and NTD risk, suggesting that RFC1 may not be a major determinant in folate-related NTD etiology across different populations [14,15]. In contrast, other investigations, particularly in populations with suboptimal folate status, have indicated a potential modest risk elevation linked to the G allele, possibly due to altered folate transport efficiency that impairs cellular folate availability during early embryogenesis [16,17].

The RFC1 gene encodes a transmembrane protein crucial for transporting folate, which is essential for intracellular folate levels and normal cellular metabolism. Folate is a key precursor in purine and pyrimidine synthesis, necessary for DNA replication, repair, and methylation. Variants like the A80G polymorphism in RFC1 gene can reduce folate transport efficiency, leading to impaired nucleotide synthesis, disrupted methylation, and genomic instability. These effects are particularly critical during embryogenesis, increasing the risk of NTDs and other developmental abnormalities. Elevated homocysteine levels due to impaired folate metabolism may further contribute to oxidative stress and complications [14].

The importance of RFC1 extends beyond metabolic pathways. The absorption of folate and its cellular uptake, facilitated by the RFC1 gene encodes a cell surface transmembrane protein that facilitates the bidirectional movement of folate across cell membranes [14,18].

RFC1 gene polymorphisms have been associated with several disease conditions including congenital anomalies like NTDs, ventricular septal defects (VSD), autistic spectrum disorders [19], congenital heart disease (CHD) and orofacial defects [20]. The rs1131596 and rs1051260 SNPs within the RFC1 gene have received attention due to their potential influence on RFC1 function and one-carbon metabolism. However, a conclusive understanding of the impact of these particular polymorphisms on NTD susceptibility remains elusive. In this study, we aimed to assess the contribution of RFC1 polymorphisms at rs1131596 and rs1051266 in relation to the occurrence of NTDs within an Ethiopian population.

2. Materials and Methods

2.1. Study Setting

A hospital-based case-control study was conducted in two purposely selected hospitals (Zewditu Memorial Hospital and St. Peter's Specialized Hospital) in Addis Ababa, Ethiopia. These two hospitals are among the leading referral hospitals in Addis Ababa with specialized pediatric neurosurgery departments. Infants and children affected by neural tube defects are referred to these

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centers from Addis Ababa and surrounding regions across Ethiopia. The study period was from April 2022 to September 2024. Cases were those mothers having offspring who are affected with neural tube defects. Cases with any ambiguous diagnosis or multiple congenital anomalies and any other congenital anomalies other than NTDs were excluded.

2.2. Data Collection

Data collection for this study was done by trained General Practitioners (GPs) and nurses using a pretested questionnaire for comprehensive information gathering. These healthcare professionals interviewed mothers and reviewed their medical records to confirm the diagnosis of NTDs in the mothers designated as cases. The absence of NTD anomalies was also explored in the control mothers' i.e mothers having normal children and visiting the hospitals for immunization or circumcision.

In parallel, whole blood samples from the mothers were collected by senior laboratory technicians following specialized training designed to ensure the consistency of sample collection. The data collectors utilized the Open Data Kit (ODK), which was installed on encrypted tablets for conducting interviews and recording responses. This approach was implemented to prioritize data integrity, privacy and security.

Participants were recruited using a consecutive sampling approach during the defined data collection period. All eligible mothers who attended the selected hospitals and met the inclusion criteria were invited to participate in the study until the end of the recruitment period. This approach allowed the inclusion of all available cases of mothers with NTD affected children presenting to the study sites, along with controls recruited from the same hospitals.

2.3. Ethical Approval and Consent for Research Participation

Ethical clearance was obtained from the National Research Ethics Review Board under the Ministry of Education, Ethiopia (IRB 17/246/818/22, 27/10/2022), Addis Ababa University (IRB/02/14/2021, 28/09/21) and Oklahoma State University (IRB-24-234, 05/08/2024).

Participants were provided with an electronic information sheet and informed consent form using the ODK tool. Only individuals who willingly agreed to participate and formally signed the consent form were included in the research. The study was conducted in accordance with the ethical principles outlined in the Declaration of Helsinki

2.4. Blood Collection for Genetic Analysis

Whole blood samples (3 ml) were systematically collected from mothers having infants and children affected with NTDs and from a control group of mothers with unaffected infants and children and stored in EDTA tubes. These blood samples were stored under -80 degrees in an EPHI laboratory until shipped to Oklahoma State University in the United States for genetic analysis. A total of 250 maternal samples were analyzed by PCR and DNA sequencing was done for RFC1 SNPs rs1131596 and rs1051266.

2.5. DNA Extraction and Genotyping

Genomic DNA was extracted from 50 µL of whole blood using the HighPrepTM Blood & Tissue DNA Kit (MagBio Genomics, Gaithersburg, MD) according to manufacturer instructions. Genomic DNA was stored at -20°C until used for PCR. Genomic DNA was amplified by PCR using primers (5′-gtgaagttcttgtcgggccccaggaggtag-3′ and 5′-gcccagcagtgccatgagtctagtg-3′) designed by Primer3 software [21] and GoTaq Green polymerase (Promega, Madison, WI). PCR conditions were as follows: an initial denaturation at 95 degrees for 2 minutes, then 35 cycles of 95 degrees for 30 seconds and annealing and extension at 68 degrees for 1 minute (step 3), and a final extension at 72 degrees for 5 minutes. Samples were sequenced by Eurofins Genomics (Louisville, KY) and chromatograms were viewed individually for RFC1 SNPs rs1131596 and rs1051266, similar to methods published by our laboratory previously [22].

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2.6. Statistical Analysis

Genotype and allele frequencies were calculated for both SNPs (rs1131596 and rs1051266), and Hardy–Weinberg equilibrium (HWE) was assessed to evaluate genotype distribution within the study population. Minor allele frequency (MAF) was computed and compared with African population MAF data reported in the ALFA Allele Frequency Aggregator from the NCBI database [23,24].

Associations between maternal genotypes and the risk of NTD-affected births were assessed using logistic regression. Regressions were applied under an additive genetic model, treating the number of minor alleles as a continuous predictor, and a recessive genetic model, comparing individuals homozygous for the minor allele with all others. All logistic regression analyses were performed without covariates. All statistical analyses were conducted using SPSS version 24, with statistical significance set at p-value less than 0.05.

3. Results

3.1. Participants' Characteristics

The study enrolled 250 mothers with a case to control ratio of one to three. Among the cases, 55.6% were mothers having children with spina bifida and 44.4% were those having hydrocephalus. Among all participants 82.4% were in the age group of 20-34 years whereas 16% were beyond 35 years of age. The proportion of the mothers at the age of 35 and above constituted 17.5% of cases and 15.5% of controls respectively.

Among the study participants, 91.6% of the mothers were living in urban areas and 8.4 % were from rural areas. Most of the participants were literate; 54.8% had completed high school and above and only 8% had no formal education. Nearly two thirds (62%) of the women were housewives.

Table 1. Socio-demographic characteristics of mothers with and without a prior NTD birth.

Characteristics	Case (n = 63)	Control (n = 187)	Total (n = 250)	χ^2	p-value
Maternal age (years)				1.34	0.935
16 - 20	1 (1.6%)	3 (1.6%)	4 (1.6%)		
20–34	51 (81.0%)	155 (82.9%)	206 (82.4%)		
35 - 45	11 (17.5%)	29 (15.5%)	40 (16.0%)		
Residence				12.40	<0.001
Urban	51 (81.0%)	178 (95.2%)	229 (91.6%)		
Rural	12 (19.0%)	9 (4.8%)	21 (8.4%)		
Marital status				1.33	0.249
Unmarried	2 (3.2%)	2 (1.1%)	4 (1.6%)		
Married	61 (96.8%)	185 (98.9%)	246 (98.4%)		
Educational status				5.53	0.137
No formal education	7 (11.1%)	13 (7.0%)	20 (8.0%)		
Primary school	23 (36.5%)	70 (37.4%)	93 (37.2%)		
Secondary school	30 (47.6%)	76 (40.6%)	106 (42.4%)		
Higher education and above	3 (4.8%)	28 (15.0%)	31 (12.4%)		
Occupation				47.41	<0.001
Housewife	62 (98.4%)	93 (49.7%)	155 (62.0%)		
Employed/business	1 (1.6%)	94 (50.3%)	95 (38.0%)		
Monthly income				26.70	<0.001
Low	40 (63.5%)	51 (27.3%)	91 (36.4%)		

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Lower-middle	23 (36.5%)	136 (72.7%)	159 (63.6%)		
Religion				4.52	0.341
Orthodox	48 (76.2%)	136 (72.7%)	184 (73.6%)		
Muslim	13 (20.6%)	29 (15.5%)	42 (16.8%)		
Protestant	2 (3.2%)	20 (10.7%)	22 (8.8%)		
Number of children				1.21	0.750
One	35 (55.6%)	114 (61.0%)	149 (59.6%)		
Two	24 (38.1%)	58 (31.0%)	82 (32.8%)		
≥Three	4 (6.4%)	15 (8.0%)	19 (7.6%)		

3.2. Detection of rs1131596 & rs1051266 SNPs in RFC1 Gene Among Ethiopian Mothers

RFC1 genotypes were tested for Hardy-Weinberg Equilibrium (HWE) and all the three genotypes for both SNPs had substantial deviation from HWE. As shown in Table 2, the chi-square results are very high and the p values are far below 0.05 in the controls, indicating that the genotype distributions significantly deviate from HWE.

Table 2. Hardy–Weinberg equilibrium analysis of RFC1 SNPs among case and control mothers.

SNP	Genotype	Cases (n = 63) Observed	Expected	Controls (n = 187) Observed	Expected	χ^2 (Case)	χ^2 (Control)	p-value (Case)	p-value (Control)
rs1131596 (−43T>C)	TT	48 (76.2%)	42.36	134 (71.7%)	108.01	0.75	6.25	<0.001	<0.001
	TC	7 (11.1%)	18.59	19 (10.2%)	68.21	7.23	35.50		
	CC	8 (12.7%)	2.04	34 (18.2%)	10.77	17.41	20.07		
	Total χ^2					25.39	61.82		
rs1051266 (80A>G)	AA	36 (57.1%)	33.58	103 (55.1%)	87.62	0.18	2.55	0.127	<0.001
	AG	20 (31.7%)	24.82	50 (26.7%)	80.76	0.94	11.06		
	GG	7 (11.1%)	4.60	34 (18.2%)	18.62	0.52	3.96		
	Total χ^2					1.64	18.74		

For the RFC1 gene polymorphism rs1131596 (−43T>C), the combined prevalence of the heterozygous (TC) and homozygous variant (CC) genotypes was 27.2%, with 10.4% heterozygous and 16.8% homozygous individuals. Accordingly, the wild-type (TT) genotype accounted for the remaining 72.8% of the study population. Similarly, for rs1051266 (80A>G), the prevalence of heterozygous (AG) and homozygous variant (GG) genotypes was 28.0% and 16.4%, respectively, yielding a cumulative variant frequency of 44.4% and a wild-type (AA) prevalence of 55.6%. The presence of maternal RFC1 polymorphisms at either locus did not demonstrate a statistically significant association with a higher risk of NTD affected births ($p = 0.601$ and $p = 0.225$, respectively).

The mean allele frequency (MAF) for rs1131596 reference allele in women with prior NTD births was 0.818 whereas the MAF in control women without NTD births was 0.767 (Table 3). For 1000

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genomes and the African population, the mentioned MAF for the alternate (risk) allele for rs1131596 was A=0.280 whereas MAF for rs1051266 was C=0.327 [23,24].

Table 3. Allele frequency of selected RFC1 SNPs of the Ethiopian mothers compared to the average for the African population according to Allele Frequency Aggregator (ALFA).

SNP	Population	Group	Reference allele	Alternate allele
rs1131596	Ethiopia (n = 250)	Cases	G = 0.818	A = 0.182
		Controls	G = 0.767	A = 0.232
	Africa (n = 1322)	—	G = 0.719	A = 0.280
rs1051266	Ethiopia (n = 250)	Cases	T = 0.762	C = 0.238
		Controls	T = 0.685	C = 0.315
	Africa (n = 1322)	—	T = 0.673	C = 0.327

3.3. Maternal Polymorphisms in RFC1 Gene and Neural Tube Defect

As shown in Table 4, among mothers included in the study, those carrying the TC and CC genotypes of RFC1 SNP rs1131596 exhibited no substantial increase in the incidence of offspring with NTDs compared to those with the TT genotype, as evidenced by a statistically insignificant p-value of 0.605 and an odds ratio of 0.657.

Even though mothers carrying the AG and GG genotypes of RFC1 SNP rs1051266 have higher proportion, there is still no significant association found in comparison with the reference genotype (AA) which constitutes 55.6% of the total population (p = 0.425) (Table 4). Therefore, in both rs1051266 and rs1131596 polymorphism, a statistically significant difference was not observed among mothers with and without prior NTD.

Table 4. Distribution of rs1131596 & rs1051266 genotypes and alleles among women with and without a prior NTD birth using additive model.

SNP / Genotype	Total (n = 250)	Cases (n = 63)	Controls (n = 187)	p-value	OR	95% CI
rs1131596 (−43T>C)						
TT (Reference)	182 (72.8%)	48 (76.2%)	134 (71.7%)	0.605	—	—
TC	26 (10.4%)	7 (11.1%)	19 (10.2%)	0.953	1.029	0.407–2.599
CC (Alternate)	42 (16.8%)	8 (12.7%)	34 (18.2%)	0.325	0.657	0.284–1.518
C allele	110 (22.0%)	23 (18.2%)	87 (23.2%)	—	—	—
T allele	390 (78.0%)	103 (81.8%)	287 (76.7%)	—	—	—
rs1051266 (80A>G)						
AA (Reference)	139 (55.6%)	36 (57.1%)	103 (55.1%)	0.425	—	—
AG	70 (28.0%)	20 (31.7%)	50 (26.7%)	0.681	1.144	0.602–2.176
GG (Alternate)	41 (16.4%)	7 (11.1%)	34 (18.2%)	0.248	0.589	0.240–1.445
A allele	313 (72.8%)	94 (76.2%)	256 (68.5%)	—	—	—
G allele	117 (27.2%)	34 (23.8%)	118 (31.5%)	—	—	—

Logistic regression was performed without covariates (unadjusted model). Odds ratios (ORs) and 95% confidence intervals (CIs) represent crude associations between genotype categories and NTD outcome.

3.4. RFC1 Gene Polymorphism (rs1131596 & rs1051266) Using the Recessive Genetic Model

We investigated the role of rs1131596 & rs1051266 in RFC1 gene using a recessive genetic model, where mothers of homozygous carriers of the variant allele were compared with other genotypes

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combined (heterozygous and homozygous wild-type) to assess the potential association with the occurrence of NTD affected births.

The distribution of genotypes under the recessive model, with allele frequencies and odds ratios calculated for homozygous variant carriers relative to the combined group of heterozygous and homozygous wild-type individuals are summarized in Table 5. More than 16% of the participants were homozygous carriers for both rs1131596 & rs1051266 SNPs. However, the proportion of mothers with the CC genotype/ homozygous carrier (18.2%) without prior NTD affected births was higher compared to those mothers with NTD births (12.7%) in rs1131596.

Using logistic regression, we observed that mothers homozygous for the variant genotype CC, in the recessive model for rs1131596 showed a less risk of having NTD affected births (OR =0.654, 95% CI: 0.285 – 1.50, p = 0.317). This finding suggests that maternal homozygosity (carrying the risk alleles) for rs1131596 polymorphism in RFC1 gene doesn't have relevant association with NTD affected births.

Similarly, the risk genotype GG has shown a higher proportion in control mothers than cases in rs1051266 polymorphism. Mothers carrying homozygous variants of rs1051266 polymorphism in RFC1 gene are not likely to develop NTD affected births (OR = 0.562, 95% CI: 0.562 – 4.240, p = 0.194). Hence, this p value is not high enough to suggest extra risk for NTD from having the homozygous variants.

Table 5. Genotype and Alleles for selected SNPs of RFC1 variant (rs1131596 & rs1051266) among women with and without a prior NTD birth using recessive model.

SNP / Genotype	Total (n = 250)	Cases (n = 63)	Controls (n = 187)	p-value	OR	95% CI
rs1131596 (–43T>C)						
TT (Reference)	182 (72.8%)	48 (76.2%)	134 (71.7%)	0.605	—	—
TC	26 (10.4%)	7 (11.1%)	19 (10.2%)	0.953	1.029	0.407–2.599
CC (Alternate)	42 (16.8%)	8 (12.7%)	34 (18.2%)	0.325	0.657	0.284–1.518
C allele	110 (22.0%)	23 (18.2%)	87 (23.2%)	—	—	—
T allele	390 (78.0%)	103 (81.8%)	287 (76.7%)	—	—	—
rs1051266 (80A>G)						
AA (Reference)	139 (55.6%)	36 (57.1%)	103 (55.1%)	0.425	—	—
AG	70 (28.0%)	20 (31.7%)	50 (26.7%)	0.681	1.144	0.602–2.176
GG (Alternate)	41 (16.4%)	7 (11.1%)	34 (18.2%)	0.248	0.589	0.240–1.445
A allele	313 (72.8%)	94 (76.2%)	256 (68.5%)	—	—	—
G allele	117 (27.2%)	34 (23.8%)	118 (31.5%)	—	—	—

Logistic regression was performed without covariates (unadjusted model). Odds ratios (ORs) and 95% confidence intervals (CIs) represent crude associations between genotype categories and NTD outcome.

4. Discussion

In the present study, the association of RFC1 polymorphism (rs1131596 and rs1051266) was assessed in mothers who had prior NTD births and those with no prior NTDs births. The association of these polymorphisms with socio-demographic, clinical and nutritional risk factors was assessed. We found no association between the presence of maternal RFC1 rs1131596 and rs1051266 polymorphisms and increased risk for NTD births which is in agreement with previous studies [25–27]. However, this study provides important genetic epidemiological data from Ethiopia, a

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population with one of the highest reported neural tube defect burdens globally and where genetic studies of folate metabolism pathways remain scarce.

We originally hypothesized that genetic variation in either of these two RFC1 polymorphisms would be associated with a greater risk of offspring NTDs. However, no significant association between these two polymorphisms in the mother and NTDs in the infant were found. The rs1051266 variant is the most studied RFC1 polymorphism. It has been associated with an increased risk of NTDs in some populations, although results vary across populations and studies [16,28].

A meta-analysis of 85 case-control comparisons on 5 common genetic variants found a suggestive but not a confirmed association of RFC-1 SNPs with increased risk of NTDs [19]. Another recent meta-analysis of 42 studies found a possible link between RFC-1 A80G and the risk of NTDs but it was only confirmed for Asian ethnicity, potentially because of the contradictory results in studies performed in the Caucasian population [20]. However our analysis revealed no significant association between the GG, AG and AA genotype ($p = 0.605$), and the risk of NTDs, indicating that the RFC1 80A>G polymorphism does not appear to influence susceptibility. This lack of association is consistent with previous studies [25,27,29].

In contrast, a study by Cao and colleagues showed a statistical difference in the allele and genotype frequencies of rs1051266 in RFC1 gene between cases and controls and the risk for NTDs was also higher in children with G allele and GG genotype, compared with A allele and AA genotype, respectively [29]. Studies suggested that G allele is associated with reduced folate transport efficiency, leading to lower intracellular folate levels [16,30]. This folate deficiency can impair nucleotide biosynthesis and methylation reactions, which are critical for neural tube closure during embryogenesis. Similarly, a study conducted on the Han population in Northern China reported a significant correlation between a RFC1 polymorphism (rs1051266) and increased NTD risk, particularly implicating the G allele [29].

Similarly, this polymorphism was associated with NTD susceptibility in both Italian [16] and Polish populations [17] but not Irish population [31](28) and Ethiopian population [15] suggesting that the genetic influence of RFC1 polymorphisms or additional factors may vary across different populations. This health impact associated with rs1051266 is reportedly due to Arg27His amino acid substitution which may impair folate transport efficiency that occurs when the G allele is substituted by A in an RFC1 polymorphism. The A allele (His27) is associated with reduced folate transport activity, potentially leading to lower intracellular folate levels and elevated homocysteine, a known teratogen that disrupts endothelial function and contributes to neural tube defects. Additionally, the Arg-to-His substitution may affect RFC1 protein stability and membrane localization, further reducing folate uptake and increasing NTD risk [16].

There are also a few studies indicating increased risk of congenital anomalies like NTD births [29] orofacial and conotruncal heart defects in the presence of RFC1 A80G polymorphism [26,32,33]. These divergent results may indicate gene–nutrient interactions, variation in ethnicity, sample size limitations among cases and controls, or methodological difference across studies.

Unlike rs1051266 SNP in RFC1, the effect of rs1131596 polymorphism has not to our knowledge been studied in relation to NTD. However, this SNP located in the SLC19A1 gene and encoding the RFC1 protein has shown variable association result with several other disease conditions like recurrent pregnancy loss [34], leukemia [35], ischemic stroke, silent brain infarction [36] and oral cavity cancers [37]. Notably, rs1051266 (A80G), a missense variant altering the protein sequence (His > Arg), is in strong linkage disequilibrium (LD) with rs1131596 ($r^2 = 0.98$) [7]. Thus, any observed associations in other studies on rs1131596 may stem from its LD with rs1051266. The indirect implication of rs1131596 in folate metabolism, through its linkage to rs1051266, suggests a potential association with NTDs. While direct evidence linking rs1131596 to NTDs is lacking, its role in altering folate metabolism provides a plausible basis for its potential contribution to NTD risk.

In this study, maternal age, education, and household economic status showed no significant association with neural tube defects (NTDs), aligning with findings from local case-control studies in Eastern Ethiopia [37] and at Debre Berhan Specialized Hospital [38], which similarly reported no

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significant relationships after adjusting for confounders. These results are also consistent with evidence from a large multi-centered U.S. case-control study [39], which found no association between socioeconomic factors, including maternal age, education, and income, with the occurrence of NTDs. However, some of the studies in a meta-analysis showed contradictory result i.e an increased risk of congenital anomalies like NTD to women living in deprived neighborhoods measured according to income, education and occupation [40]. These findings underscore the multifactorial nature of risk for NTD with genetic predispositions, folate metabolism disruptions, and environmental factors playing more decisive roles than demographic variables [17,26,33,40,41].

The expected HWE was not observed in the control group for both RFC1 polymorphisms. Thus, these results should be interpreted with caution, because case-control studies assume the existence of HWE equilibrium at least in controls. The observed HWE disequilibrium may be due to random selection of samples, model, or disease complexity.

Although no significant association was observed, this study provides important genetic epidemiological data from Ethiopia, a population with one of the highest reported neural tube defect burdens globally[8,42] [8,9] and where genetic studies of folate metabolism pathways remain scarce

4.1. Strengths of the Study

This study has several notable strengths. The case-control design is appropriate for investigating NTDs, which are relatively rare but carry substantial morbidity and mortality, allowing efficient comparison between affected and unaffected births. The use of robust molecular techniques, including PCR and genomic sequencing, enhanced the accuracy of polymorphism detection and minimized genotyping error.

Although no significant association was observed between NTDs and the selected RFC1 polymorphism, the study identified a high frequency and diversity of genetic variants, providing important baseline evidence of genetic heterogeneity that may contribute to NTD risk. Notably, this is the first study in Ethiopia to examine these polymorphisms using maternal whole-blood DNA, adding novel and valuable data to the local genetic research landscape.

Conducting the study in Ethiopia, a country with one of the highest reported burdens of NTD-related mortality globally, further strengthens its public health relevance. Studying genetic polymorphisms in such a high-burden context enhances the relevance of the findings and supports the need for continued genetic and nutritional research to inform prevention strategies.

4.2. Limitations of the Study

This study has some limitations that should be considered when interpreting the findings. First, the relatively small sample size, particularly the limited number of cases compared with controls, may have reduced the statistical power to detect modest genetic associations. In addition, while the present study focused on selected polymorphisms using targeted molecular approaches, future studies incorporating larger sample sizes and whole-genome or exome sequencing would allow a more comprehensive assessment of genetic variation and may better capture the complex genetic architecture underlying NTDs.

5. Conclusion

Although this study did not identify a statistically significant association between maternal RFC1 gene polymorphisms and the occurrence of NTD-affected births, the findings do not eliminate the possibility of a genetic contribution. Given the complex and multifactorial nature of NTDs, the role of RFC1 variants may be subtle or context-dependent, requiring more sensitive or comprehensive approaches to detect.

To gain a more complete understanding of the genetic architecture underlying NTDs in the Ethiopian population, further investigations are necessary. Advanced genomic techniques such as whole-genome sequencing of RFC1 and other genes involved in folate metabolism could help

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identify rare or interacting variants that contribute to NTD susceptibility. Such efforts are crucial for informing future prevention strategies and tailoring interventions to high-risk groups.

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Institutional Review Board Statement: Ethics Approval: The study was conducted in accordance with the Declaration of Helsinki, and the study protocol received ethical approval from the Institutional Review Boards of Addis Ababa University (IRB No. 04-14-2021) and Oklahoma State University (IRB No. 24-234). As the biological samples were shipped from Ethiopia to OSU, USA, material transfer agreement and additional ethical approval was obtained from the National Research Ethics Review Board of the Ministry of Education, Ethiopia (IRB No. 17/246/818/22).

Prior to study initiation, formal authorization was obtained from the Chief Executive Officer of each participating health facility. Approval letters included detailed descriptions of the study objectives, data collection procedures, and participant information sheets, together with requests to identify eligible participants and to access the respective hospital departments including the pediatric neurosurgery. Institutional permission was subsequently granted by the respective, confirming adherence to ethical standards and regulatory requirements. All participants were recruited following a written informed consent process conducted in accordance with approved ethical guidelines.

Informed Consent Statement: Written informed consent was obtained from all participants prior to their inclusion in the study. Participant data were anonymized and accessible only to the principal investigator where the other authors had access only to the de-identified one. Consent was obtained from all mothers for participation in the interview questionnaire, collection and genetic analysis of blood samples, as well as publication of study findings in scientific journals.

Data Availability Statement: Ethical and legal restrictions specified in the approved protocol preclude unrestricted public deposition of the individual-level data. De-identified data, including chromatograms, and summary statistics not presented in the manuscript may be made available upon reasonable request to the first author, contingent on approval by the relevant ethics committee and the execution of a data-transfer agreement. Requests should be directed to the first author and will be evaluated on a case-by-case basis.

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Abbreviations

The following abbreviations are used in this manuscript:

AAU	Addis Ababa University
ALFA	Allele Frequency Aggregator
EPHI	Ethiopian Public Health Institute
HWE	Hardy–Weinberg Equation
MAF	Mean Allele Frequency
MTHFR	Methylenetetrahydrofolate Reductase
NCBI	National Center for Biotechnology Information
NTD	Neural Tube Defect
ODK	Open Data Kit
OSU	Oklahoma State University
PCR	Polymerase Chain Reaction
RFC	Reduced Folate Carrier
SNP	Single Nucleotide Polymorphism

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