applied sciences

MDPI

Article

The Importance of Gene Variants Related to Folate Uptake and Distribution in the Etiology of Recurrent Miscarriage and Intrauterine Fetal Demise

Martyna Kozłowska-Wytyk [1], Grażyna Kurzawińska [2], Marcin Ożarowski [3,*], Aleksandra E. Mrozikiewicz [4], Piotr Olbromski [5], Tomasz M. Karpiński [6], Bogusław Czerny [3,7] and Hubert Wolski [8]

1 Division of Obstetrics and Gynecology, Holy Family Hospital, Jarochowskiego 18, 60-235 Poznan, Poland; mkw.ginekolog@gmail.com
2 Laboratory of Molecular Biology, Division of Perinatology, Poznan University of Medical Sciences, Polna 33, 60-535 Poznan, Poland; gkurzawinska@ump.edu.pl
3 Institute of Natural Fibres and Medicinal Plants, Wojska Polskiego 71b, 60-630 Poznań, Poland; boguslaw.czerny@iwnirz.pl
4 Department of Reproduction, Poznan University of Medical Sciences, Polan 33, 60-535 Poznań, Poland; mrozikiewicz@gmail.com
5 Clinic of Operational Gynecology, Poznan University of Medical Sciences, Polna 33, 60-535 Poznan, Poland; olbromski.piotr@gmail.com
6 Chair and Department of Medical Microbiology, Poznań University of Medical Sciences, Rokietnicka 10, 60-806 Poznań, Poland; tkarpin@ump.edu.pl
7 Department of General Pharmacology and Pharmacoeconomics, Pomeranian Medical University in Szczecin, Żołnierska 48, 70-204 Szczecin, Poland
8 Institute of Medical Sciences, Academy of Applied Sciences, Kokoszków 71, 34-400 Nowy Targ, Poland; hubertwolski@wp.pl
* Correspondence: marcin.ozarowski@iwnirz.pl

check for updates

Citation: Kozłowska-Wytyk, M.; Kurzawińska, G.; Ożarowski, M.; Mrozikiewicz, A.E.; Olbromski, P.; Karpiński, T.M.; Czerny, B.; Wolski, H. The Importance of Gene Variants Related to Folate Uptake and Distribution in the Etiology of Recurrent Miscarriage and Intrauterine Fetal Demise. *Appl. Sci.* **2024**, *14*, 11847. https://doi.org/10.3390/app142411847

Academic Editor: Gemma Leone

Received: 16 September 2024
Revised: 17 November 2024
Accepted: 13 December 2024
Published: 18 December 2024

Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

Abstract: (1) Background: The study involves an assessment of the frequency of selected gene variants related to folate uptake and distribution (*FOLR1* rs2071010, rs630074, *FOLH1* rs61886492, *GGH* rs11545078, rs3758149 and *SLC19A1* rs1051266) in a group of women with fetal demise in the Polish population. (2) Methods: A total of 310 subjects were enrolled in the study. There were 110 females with idiopathic recurrent miscarriages (RM), 80 with stillbirth (IUFD) and 120 healthy controls. Designated SNVs were determined by using PCR-RFLP methods. The difference in fetal demise prevalence was assessed using a chi-square test and logistic regression analysis. (3) Results: The rs630074 variant of the *FOLR1* gene is associated with a statistically significant increase in the risk of IUFD in a recessive model (OR = 2.03, 95%CI: 1.06–3.90, p = 0.033). The rs61886492variant f *FOLH1* is linked to an increased risk of IUFD in co-dominant (p = 0.030), dominant (OR = 2.62, 95%CI: 1.07–6.38, p = 0.032) and log-additive models (OR = 2.64, 95%CI: 1.15–6.06 p = 0.030). In female carriers of the *A* allele, the risk of IUFD was 2.8 times higher compared to the control group. No relationship between the mother's genotype and the newborn's birth weight or placental weight was observed for the studied SNVs. (4) Conclusions: Our study finds that the rs61886492 variant of the FOLH1 gene is associated with IUFD in Polish women. However, pregnancy failures have a multifactorial pathology and other genetic or environmental factors may also contribute to their complex etiology. Further research, preferably with larger groups of women from different ethnic backgrounds, is needed to confirm the results of the current study.

Keywords: recurrent miscarriage; folate uptake and distribution; gene variants

1. Introduction

Fetal demise can occur at any stage of pregnancy and may result from maternal, fetal, or placental factors. Pregnancy loss is categorized as miscarriage or stillbirth, depending on the timing of the loss. Definitions for these terms vary, with gestational age or fetal weight

serving as key criteria. However, gestational age is considered a more reliable indicator of fetal maturity and viability compared to birth weight [1]. According to the European Society of Human Reproduction and Embryology, miscarriage is defined as pregnancy loss before 22 weeks of gestation, whereas the World Health Organization (WHO) defines it as the expulsion or removal of a fetus (or embryo) weighing less than 500 g, which aligns with approximately 22 weeks of gestation. Around 1–2% of couples attempting conception face unexplained recurrent pregnancy loss (uRPL), characterized by two or more spontaneous miscarriages without an identifiable cause [2,3]. Intrauterine fetal demise (IUFD), also referred to as stillbirth, describes fetal death occurring at or beyond the 22nd week of gestation [4]. Despite progress in understanding its causes, a significant proportion of stillbirths remain unexplained.

Folates play a crucial role in amino acid and nucleic acid metabolism as well as in one-carbon metabolic pathways. It is widely recommended for women of reproductive age, pregnant individuals, and those with MTHFR polymorphism to take folate supplements. A daily intake of 400–800 mcg of folate is advised to reduce the risk of neural tube defects [5,6].

Folic acid (FA), a synthetic form of vitamin B9, is commonly added to fortified foods and supplements. Within the body, it must be converted into the bioactive folate derivative (6S)-5-MTHF through cellular metabolism. Supplementing with (6S)-5-MTHF during pregnancy offers advantages over folic acid because it circumvents the metabolic block caused by enzyme polymorphisms, reducing the likelihood of unmetabolized folic acid (UMFA) accumulation [6]. Concerns regarding FA and its connection to UMFA syndrome have grown, as UMFA has been detected in umbilical cord blood and even in infants' circulation [7].

The bioavailability of folates depends not only on dietary intake but also on their absorption and transport within the body. During pregnancy, efficient folate transfer across the placenta is critical for fetal development. Folate transport primarily involves three key transporter types: the reduced folate carrier (RFC), high-affinity folate receptors (FOLRs), and the proton-coupled folate transporter (PCFT). These transporters are expressed in the human placenta, although their exact localization remains uncertain [8]. It is hypothesized that during pregnancy, when folate requirements are elevated, mutations in genes encoding folate transporters or receptors in either the mother or fetus may worsen folate deficiency or create a deficiency-like state, even with adequate folate intake [9].

The reduced folate carrier protein 1 (RFC1), encoded by the SLC19A1 gene (GeneID: 6573), plays a key role in regulating intracellular folate concentrations and is prominently expressed in tissues such as the liver and placenta [10]. Structurally, *SLC19A1* belongs to the major facilitator superfamily (MFS), consisting of twelve transmembrane segments with both the N- and C-terminal regions positioned in the cytoplasm. Its transport activity relies on the outward movement of anionic metabolites along their concentration gradients to drive folate uptake [11]. The most studied single nucleotide polymorphism (SNP) in this gene is 80A>G (rs1051266), which results in a histidine-to-arginine substitution in exon 2, influencing folate uptake efficiency and altering metabolic pathways [12,13]. Additionally, two other polymorphisms, -43T>C (rs1131596) near the transcription start site and 696C>T (rs12659) within the coding region, show strong linkage disequilibrium with the 80A>G variant [14,15]. These genetic variations, including the 80A>G SNP, may increase susceptibility to pregnancy loss in certain populations [15,16].

Folate transport from the maternal intestine to the embryo depends on both transporters and folate receptors (FRs) [9]. The FR family comprises four isoforms: *FRα* (257 aa, 30 kDa), *FRβ* (255 aa, 29 kDa), *FRγ* (245 aa, 28 kDa), and *FRδ* (250 aa, 28.6 kDa), encoded by the genes *FOLR1* (Gene ID: 2348), *FOLR2* (Gene ID: 2350), *FOLR3* (Gene ID: 2352), and *FOLR4* (Gene ID: 390243), respectively. These proteins, also called folate-binding proteins (FBPs), bind folic acid, 5-mTHF, and folate-conjugated compounds with high affinity, facilitate cellular uptake via receptor-mediated endocytosis. *FRα*, *FRβ*, and *FRδ* are GPI-anchored membrane proteins, whereas *FRγ* lacks the GPI anchor and is secreted. Among these isoforms, *FRα*, encoded by *FOLR1*, is the most extensively studied [17–19].

Research in mice highlights FOLR1's critical role during early development, as Folr1 knockout embryos exhibit severe defects and do not survive [20]. The *FOLR1* gene spans 6.7 kb and contains seven exons [21]. The human folate receptor genes and *FOLH1* (Gene ID: 2346), which encodes glutamate carboxypeptidase II (GCPII, EC 3.4.17.21), are located on chromosome 11. GCPII facilitates folate absorption by converting folylpolyglutamate into folymonoglutamate. The C>T variant (rs61886492) in *FOLH1*, resulting in the substitution of histidine with tyrosine at position 475 (H475Y), reduces GCPII activity, leading to lower blood folate levels and elevated homocysteine concentrations.

Intracellular folate homeostasis is regulated by the combined actions of two key enzymes: folylpolyglutamate synthase (FPGS, Gene ID: 2356) and gamma-glutamyl hydrolase (GGH, Gene ID: 8836). These enzymes work together to ensure proper intracellular folate concentration and distribution. FPGS retains folates within cells by adding glutamate residues, while GGH (EC 3.4.19.9) breaks down polyglutamylated folates into monoglutamylated forms, facilitating their export from the cell [22]. A variant in the *GGH* gene (rs3758149 C allele) has been shown to increase transcriptional activity and enhance binding affinity for nuclear proteins in the CEM/C1 T lymphoblast cell line when compared to the T allele [23].

Therefore, this study investigated the relationships between the frequency of selected gene variants related to folate uptake and distribution (*FOLR1* rs2071010, rs630074, *FOLH1* rs61886492, *GGH* rs11545078, rs3758149 and *SLC19A1* rs1051266) in a group of women with RM and IUFD in the Polish population (Figure 1).

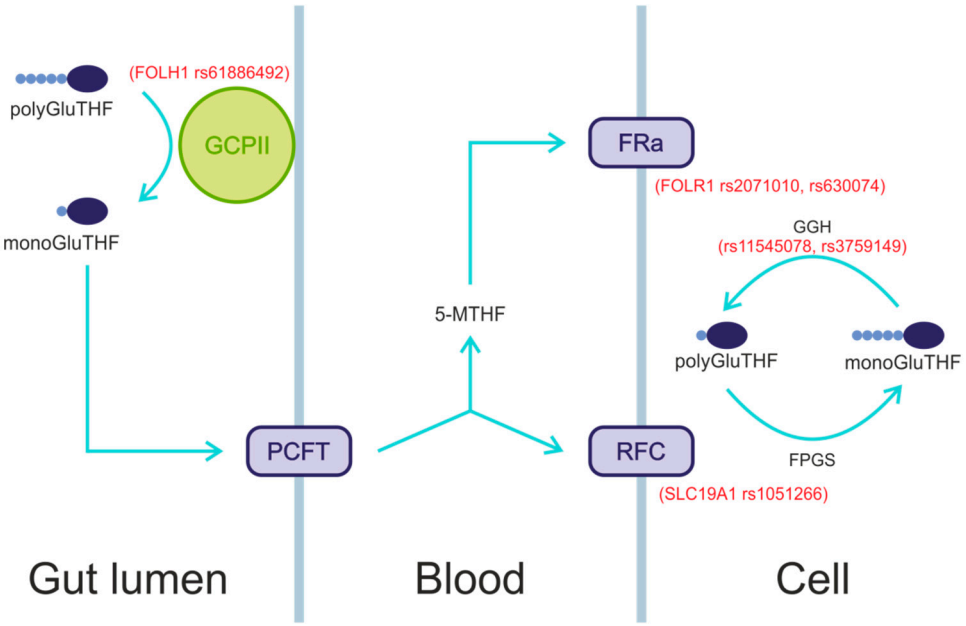


Figure 1. Schematic illustration of folate uptake and distribution. The genes studied and their polymorphic variants are marked in red.

2. Materials and Methods

2.1. Study Population

A total of 310 EDTA blood samples were obtained from the Department of Perinatology and Women Diseases of Poznan University of Medical Sciences, Poznan. These encompassed 190 women with fetal death (110 women with ≥3 idiopathic recurrent miscarriages ≤22 weeks of pregnancy with the same partner and 80 women who experienced intrauterine fetal demise of a single fetus after the 22nd week) and 120 healthy women with a history of at least two uncomplicated pregnancies resulting in the delivery of a healthy full-term newborn (≥37 week), with no history of pregnancy loss. Patients with pregnancy loss due to anatomic, hormonal, chromosomal, infectious, autoimmune, or thrombotic

causes were excluded from the study group. No participant in this study had a history of diabetes or hypertension, or a history of drug, alcohol or smoking abuse. Stillbirth cases from multi-fetal pregnancies were excluded from our analysis. Patient blood samples were collected after pregnancy between 2020 and 2023. All patients and controls were of Polish ethnicity and declared taking 400 µg of folic acid daily during pregnancy. The study complied with ethical requirements and has been approved by the Ethics Committee of the Poznan University of Medical Sciences (No. 596/19).

2.2. Selection of the SNVs

Six single nucleotide variations (SNVs; formerly SNPs), *FOLR1* rs2071010, rs630074, *FOLH1* rs61886492, *GGH* rs11545078, rs3758149 and *SLC19A1* rs1051266 were selected for this study. The SNVs were collected from the National Centre for Biotechnology Information (NCBI) Single Nucleotide Polymorphism Database (dbSNP) [24,25]. In the 1000 Genomes Project dataset, the minor allele frequencies (MAFs) for the analyzed single nucleotide variants (SNVs) are as follows: 6–8% for allele A (rs2071010), 46–64% for allele T (rs630074), 2–7% for allele A (rs61886492), 8–14% for allele A (rs115450780), 24–30% for allele A (rs3758149), and 49–60% for allele C (rs1051266). Given the limited availability of Central European samples in the 1000 Genomes Project dataset, we applied a selection criterion of MAF > 5% based on the European populations included in the dataset. A detailed summary of the selected variants is provided in Table 1.

Table 1. Characteristics of selected genes and polymorphisms.

Gene ID	rs Number	Position (GRCh38.p14)	Region	Ref. Allele	Exonic Function	Clinical Significance in ClinVar [25]
FOLR1	rs2071010	chr11:72189920	intronic	G	—	Uncertain significance
FOLR1	rs630074	chr11:72193893	intronic	C	—	Not reported
FOLH1	rs61886492	chr11:49164722	exonic	G	H (His) > Y (Tyr)	Not reported
GGH	rs11545078	chr8:63026205	exonic	G	T (Thr) > I (Ile)	Not reported
GGH	rs3758149	chr8:63039169	upstream	G	—	Not reported
SLC19A1	rs1051266	chr21:45537880	exonic	T	H (His) > R (Arg)	Uncertain significance

GRCh38.p14.

In silico analysis was performed to predict the impact of SNVs using PredictSNV v.2 [26]. This tool identifies the functional impact of SNVs, based on the evaluation of six databases CADD, DANN, FATHMM, FitCons, FunSeq2 and GWAVA to develop category-optimal decision thresholds. The output displays the results of the five best-performing tools in neutral or deleterious form [27]. For the six SNVs tested, we obtained four neutral (rs2071010—88% expected accuracy, rs630074—77% expected accuracy, rs61886492 89% expected accuracy rs1051266—89% expected accuracy) and two deleterious effects (rs11545078—87% expected accuracy and rs3758149 with 91% expected accuracy in *GGH* gene) in the PredictSNV 2 output [26].

2.3. DNA Extraction and SNVs Genotyping

Venous blood samples from patients and control group individuals were collected into vacutainer tubes containing EDTA. Genomic DNA from the blood was extracted by using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions and DNA samples were stored at −20 °C for further analyses. The genetic variants were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay as previously described [15,28–30]. The primers for *FOLR1* gene variants rs2071010 and rs630074 were designed using the NCBI-SNV database and Primer3Plus [31]. In Table 2, the primers and restriction enzymes used for

SNV genotyping are presented. Products were electrophoresed on agarose gels with Midori Green Advanced DNA Stain (Nippon Genetics, Düren, Germany). To verify the genotyping quality for each polymorphism, 20% of random samples were genotyped. All results confirmed the preceding genotyping outcomes.

Table 2. PCR primers and restriction enzymes used for SNV genotyping.

Ref.	Fragments (bp)	Restriction Enzyme	Primer Sequences	SNV
32	G 210,160,11 A 370, 11	EcoNI	5′-CCACCTCCTCTCCCAGGTAT-3′ 5′-CAGCATCACTCTGCAAGCTC-3′	FOLR1 rs2071010
32	T 268, 127 C 395	MboII	5′-TGCCTATCCTCAAGCTTCCT-3′ 5′-CGGCCTTAAGTGCACATTTT-3′	FOLR1 rs630074
29	G 244 A 141, 103	AccI	5′-CATTCTGGTAGGAATTTAGCA-3′ 5′-AAACACCACCTATGTTTAACA-3′.	FOLH1 rs61886492
30	G 286 A 177,109	AseI	5′-GTGCCTATTTGGTTATGACA-3′ 5′-CTACTTACTAATCCTGCCCA-3′	GGH rs11545078
31	G 109, A 61, 48	BslI	5′-CGCTGCCTGGTTACCAAACT-3′ 5′-TGTTACGTCGATGTGGACTTCAG-3′	GGH rs3758149
16	T 230 C 126, 104	HaeII	5′-AGCGTCACCTTCGTCCC-3′ 5′-TCCCGCGTGAAGTTCTTG-3′	SLC19A1 rs1051266

2.4. Statistical Analysis

The data were analyzed using R software (version 4.3.1; R Foundation for Statistical Computing, Vienna, Austria) [32], along with the SNPassoc package (version 2.1-0) [33]. Results for categorical variables were expressed as frequencies and percentages, while continuous variables were presented as means ± standard deviation (SD) or medians with interquartile range, depending on their distribution. The Shapiro–Wilk test was used to assess normality, and comparisons were performed using Student's *t*-test for normally distributed data or appropriate non-parametric tests for skewed data. The Hardy–Weinberg equilibrium (HWE) was tested using the χ^2 goodness-of-fit test.

Associations between genotypes and susceptibility to intrauterine fetal demise (IUFD) and recurrent miscarriage (RM) were evaluated using logistic regression to calculate odds ratios (OR) with 95% confidence intervals (CI). The most suitable inheritance models were identified using the Akaike information criterion (AIC). Associations between genotypes and continuous variables such as newborn weight and placenta weight were examined using a univariate general linear model. Both unadjusted and confounder-adjusted statistical results are reported as mean ± SD. Statistical significance was set at $p < 0.05$.

Linkage disequilibrium (LD) and haplotype analyses were conducted using Haploview software (version 4.2) [34], which applies the expectation-maximization (EM) algorithm. Statistical power was calculated using the "genpwr" package (version 1.0.2) in R [35]. For the sample of 310 women (190 cases with fetal death and 120 controls), assuming an additive model with a case-to-control ratio of 190/310 (0.613), an alpha of 0.05, and a power of 0.80, the odds ratio was estimated at 1.75 based on the minor allele frequencies of the selected SNPs.

3. Results

3.1. Baseline Characteristics of Women

A total of 190 women diagnosed with fetal death agreed to participate in the study. For the control group, 120 women without a history of RM and IUFD were recruited. The demographic characteristics are listed in Table 3. Women with RM (32.86 ± 4.76 years) were older than the IUFD group (30.49 ± 4.50 years) and the controls (31.38 ± 3.59 years) ($p < 0.001$). There were no significant differences in maternal age between controls and IUFD ($p = 0.322$) but there was a significant difference between RM and controls ($p = 0.024$) and RM vs. IUFD ($p < 0.001$). Systolic and diastolic blood pressures (BP) differed between controls and the RM group (for SBP $p < 0.001$, and for DBP $p = 0.002$). Women from both groups with fetal demise were heavier than controls (IUFD vs. controls $p = 0.003$, RM

vs. controls $p < 0.001$) and had a higher mean BMI (20.72 ± 1.75 kg/m^2 in controls. vs. 22.55 ± 3.57 kg/m^2 in IUFD ($p < 0.001$) and 23.53±3.98 kg/m^2 in RM ($p < 0.001$)). In the control group, according to the inclusion and exclusion criteria, all women were mothers of two or more children and had no history of miscarriages or IUFD. In the IUFD group, the majority of women were childless, eighteen of them had one child, and three women had two or more children. Patients from this group had previously had miscarriages: 25% had one, 16.25% had two and 5% had three or more miscarriages (Table 3).

Table 3. The clinical characteristic of the study groups (n = 310).

Parameter	Controls (n = 120)	IUFD (n = 80)	RM (n = 110)	*p*-Value
Age (years)	31.38 ± 3.59	30.49 ± 4.50	32.86 ± 4.76	<0.001
SBP (mmHg)	100.0 [95.0; 110.0]	107.50 [100.0; 120.0]	110.0 [100.0; 120.0]	<0.001
DBP (mmHg)	62.5 [60.0; 70.0]	60.0 [60.0; 70.0]	70.00 [60.0; 80.0]	0.003
Height (cm)	166.93 ± 5.78	166.34 ± 8.28	166.94 ± 5.64	0.792
Weight (kg)	57.86 ± 6.67	62.31 ± 9.92	65.74 ± 12.41	<0.001
BMI (kg/m^2)	20.72 ± 1.75	22.55 ± 3.57	23.53 ± 3.98	<0.001
Gestational age (weeks)	39 [38; 40]	29.5 [26; 34]	14.5 [6; 18]	<0.001
Newborn weight (g)	3483.75 ± 399.83	1350.50 ± 316.45	-	<0.001
Placenta weight (g)	605 [550; 700]	412 [240; 560]	-	<0.001
Number of pregnancies	2.0 [2.0; 3.0]	2.0 [1.0; 3.0]	4.0 [3.0; 5.0]	<0.001
childless women	0 (0.00)	59 (73.75)	74 (67.27)	
healthy newborn:				<0.001
one	0 (0.00)	18 (22.50)	30 (27.27)	
≥two	120 (100.0)	3 (3.75)	6 (5.45)	<0.001
IUFD				
one	0 (0.00)	72 (90.00)	0 (0.00)	
≥two	0 (0.00)	8 (10.00)	0 (0.00)	<0.001
miscarriages				
one	0 (0.00)	20 (25.00)	0 (0.00)	
two	0 (0.00)	13 (16.25)	0 (0.00)	<0.001
≥three	0 (0.00)	4 (5.00)	110 (100.00)	

Mean ± SD, median [Q1; Q3].

3.2. Single Locus Associations

All 310 samples were successfully genotyped, and the allele frequencies of the six genetic variants showed no deviation from the Hardy–Weinberg Equilibrium (HWE) in the control group (rs2071010, $\chi^2 = 0.006$, $p = 0.937$; rs630074 $\chi^2 = 0.099$, $p = 0.951$; rs61886492, $\chi^2 = 0.182$, $p = 0.912$; rs11545078, $\chi^2 = 0.991$, $p = 0.609$; rs3758149, $\chi^2 = 2.312$, $p = 0.314$; rs1051266, $\chi^2 = 2.125$, $p = 0.345$).

The allele frequencies of the tested genetic variants were compared between groups of women with fetal demise and the controls and are presented in Table 4. In the IUFD group, a statistically significant increase in the frequency of *A* alleles of the rs61886492 variant of the *FOLH1* gene was observed compared to the controls (0.100 vs. 0.037, $p = 0.011$). In women who were carriers of the *A* allele, the risk of IUFD was 2.8 times higher compared to the control group (OR = 2.851; 95% CI: 1.227–6.624). No significant association was observed in the allelic frequencies in women with RM relative to controls. However, the distribution of the *A* allele of *FOLR1* rs2071010 was more frequent in women with RM than the controls (0.059 vs. 0.029, $p = 0.115$, OR = 2.090; 95% CI: 0.818–5.339).

Table 4. Allele frequencies of six examined polymorphisms in RM, IUFD patients and controls.

Gene/SNV	Allele	Controls (N = 240)	IUFD (N = 160)	OR (95% CI)	χ^2 *p*-Value	RM (N = 220)	OR (95% CI)	χ^2 *p*-Value
FOLR1 rs2071010	G	233 (0.971)	154 (0.963)	1.297 (0.427~3.932)	χ^2 = 0.212 0.645	207 (0.941)	2.09 (0.818~5.339)	χ^2 = 2.472 0.115
	A	7 (0.029)	6 (0.037)			13 (0.059)		
FOLR1 rs630074	C	107 (0.446)	83 (0.519)	0.746 (0.500~1.115)	χ^2 = 2.046 0.153	112 (0.509)	0.776 (0.537-1.120)	χ^2 = 1.841 0.174
	T	133 (0.554)	77 (0.481)			108 (0.491)		
FOLH1 rs61886492	G	231 (0.963)	144 (0.900)	2.852 (1.228~6.624)	χ^2 = 6.400 0.011	208 (0.945)	1.481 (0.612~3.585)	χ^2 = 0.765 0.382
	A	9 (0.037)	16 (0.100)			12 (0.055)		
GGH rs11545078	G	220 (0.917)	148 (0.925)	0.892 (0.423~1.879)	χ^2 = 0.091 0.763	207 (0.941)	0.691 (0.335~1.424)	χ^2 = 1.013 0.314
	A	20 (0.083)	12 (0.075)			13 (0.059)		
GGH rs3758149	G	173 (0.721)	119 (0.744)	0.889 (0.565~1.399)	χ^2 = 0.256 0.613	161 (0.732)	0.946 (0.628~1.427)	χ^2 = 0.070 0.792
	A	67 (0.279)	41 (0.256)			59 (0.268)		
SLC19A1 rs1051266	T	122 (0.508)	79 (0.494)	1.060 (0.711~1.582)	χ^2 = 0.082 0.775	97 (0.441)	1.311 (0.908~1.893)	χ^2 = 2.092 0.148
	C	118 (0.492)	81 (0.506)			123 (0.559)		

Pearson's *p*.

The association between selected SNVs related to folate uptake and distribution in fetal demise groups and controls was analyzed using multiple logistic regression and the results are presented in Table 5. For *FOLR1* rs630074, a statistically significant increase in the risk of IUFD was found for carriers of the *CC* genotype compared to the *TT* and *TC* genotypes in the recessive model (OR = 2.03, 95%CI: 1.06–3.90, p = 0.033, AIC = 268.7). The most interesting observations concerned the variant *FOLH1* rs61886492. A statistically significant increase in the risk of IUFD was found in co-dominant (p = 0.030, AIC = 268.5), dominant (OR = 2.62, 95%CI: 1.07–6.38, p = 0.032, AIC = 268.6) and log-additive models (OR = 2.64, 95%CI: 1.15–6.06 p = 0.030, AIC = 267.5).

Table 5. Distribution of genotypes in the study groups.

Gene, SNV	Genotypes/Model	Controls (N = 120)	IUFD (N = 80)	OR (95%CI)	*p*-Value	AIC	RM (N = 110)	OR (95%CI)	*p*-Value	AIC
FOLR1 rs2071010	GG	113 (94.2)	74 (92.5)	1.00	0.642	273.0	97 (88.2)	1.00	0.106	319.8
	GA	7 (5.8)	6 (7.5)	1.31 (0.42–4.05)			13 (11.8)	2.16 (0.83–5.64)		
	AA	0 (0.0)	0 (0.0)	—			0 (0.00)			
	Dominant	—	—	—	—	—	—	—	—	—
	Recessive	—	—	—	—	—	—	—	—	—
	Over dominant	—	—	—	—	—	—	—	—	—
	Log-additive	120 (60.0)	80 (40.0)	1.31 (0.42–4.05)	0.642	273.0	110 (47.8)	2.16 (0.83–5.64)	—	319.8
FOLR1 rs630074	TT	36 (30.0)	23 (28.8)	1.00	0.083	270.2	24 (21.8)	1.00	0.337	322.2
	TC	61 (50.8)	31 (38.8)	0.80 (0.40–1.57)			60 (54.5)	1.48 (0.79–2.76)		
	CC	23 (19.2)	26 (32.5)	1.77 (0.82–3.81)			26 (23.6)	1.70 (0.79–3.64)		
	Dominant	84 (70.0)	57 (71.2)	1.06 (0.57–1.98)	0.849	273.2	86 (78.2)	1.54 (0.84–2.79)	0.157	320.4
	Recessive	97 (80.8)	54 (67.5)	2.03 (1.06–3.90)	0.033	268.7	84 (76.4)	1.31 (0.69–2.46)	0.408	321.7
	Over dominant	59 (49.2)	49 (61.2)	0.61 (0.34–1.09)	0.092	270.4	50 945.5)	1.16 (0.69–1.95)	0.573	322.1
	Log-additive	120 (60.0)	80 (40.0)	1.31 (0.89–1.94)	0.168	271.3	110 (47.8)	1.31 (0.90–1.92)	0.162	320.5

Table 5. *Cont.*

Gene, SNV	Genotypes/Model	Controls (N = 120)	IUFD (N = 80)	OR (95%CI)	*p*-Value	AIC	RM (N = 110)	OR (95%CI)	*p*-Value	AIC
FOLH1 rs61886492	GG	111 (92.5)	66 (82.5)	1.00	0.030	268.5	98 (89.1)	1.00	0.370	321.6
	GA	9 (7.5)	12 (15.0)	2.24 (0.90–5.61)			12 (10.9)	1.51 (0.61–3.74)		
	AA	0 (0.0)	2 (2.5)	—			0 (0.0)	—		
	Dominant	9 (7.5)	14 (17.5)	2.62 (1.07–6.38)	0.032	268.6	—	—	—	—
	Recessive	120 (100.0)	78 (97.5)	—	0.159	269.5	—	—	—	—
	Over dominant	9 (7.5)	12 (15.0)	2.18 (0.87–5.44)	0.094	270.4	—	—	—	—
	Log-additive	120 (60.0)	80 (40.0)	2.64 (1.15–6.06)	0.030	267.5	110 (47.8)	1.51 (0.61–3.74)	—	321.6
GGH rs11545078	GG	100 (83.3)	69 (86.2)	1.00	0.405	272.8	97 (88.2)	1.00	0.293	321.3
	GA	20 (16.7)	10 (12.5)	0.72 (0.32–1.64)			13 (11.8)	0.67 (0.32–1.42)		
	AA	0 (0.0)	1 (1.2)	—			—	—		
	Dominant	20 (16.7)	11 (13.8)	0.80 (0.36–1.77)	0.574	272.9	—	—	—	—
	Recessive	120 (100.0)	79 (98.8)		0.400	271.4	—	—	—	—
	Over dominant	100 (83.3)	70 (87.5)	0.71 (0.32–1.62)	0.414	272.5	—	—	—	—
	Log-additive	120 (60.0)	80 (40.0)	0.89 (0.42–1.89)	0.405	273.1	110 (47.8)	0.67 (0.32–1.42)	—	321.3
GGH rs3758149	GG	59 (49.2)	43 (53.8)	1.00	0.808	274.8	61 (55.5)	1.00	0.188	321.1
	GA	55 (45.8)	33 (41.2)	0.82 (0.46–1.48)			39 (35.5)	0.69 (0.40–1.18)		
	AA	6 (5.0)	4 (5.0)	0.91 (0.24–3.44)			10 (9.1)	1.61 (0.55–4.72)		
	Dominant	61 (50.8)	37 (46.2)	0.83 (0.47–1.47)	0.525	272.8	49 (44.5)	0.78 (0.46–1.31)	0.340	321.5
	Recessive	114 (95.0)	76 (95.0)	1.00 (0.27–3.66)	1.000	273.2	100 (90.9)	1.90 (0.67–5.41)	0.222	320.9
	Over dominant	65 (54.2)	47 (58.8)	0.83 (0.47–1.47)	0.522	272.8	71 (64.5)	0.65 (0.38–1.10)	0.109	319.8
	Log-additive	120 (60.0)	80 (40.0)	0.88 (0.54–1.42)	0.599	272.9	110 (47.8)	0.94 (0.62–1.43)	0.789	322.3
SLC19A1 rs1051266	TT	35 (29.2)	23 (28.8)	1.00	0.925	275.0	28 (25.5)	1.00	0.284	321.9
	TC	52 (43.3)	33 (41.2)	0.97 (0.49–1.91)			41 (37.3)	0.99 (0.52–1.88)		
	CC	33 (27.5)	24 (30.0)	1.11 (0.53–2.33)			41 (37.3)	1.55 (0.79–3.05)		
	Dominant	85 (70.8)	57 (71.2)	1.02 (0.55–1.90)	0.949	273.2	82 (74.55)	1.21 (0.67–2.16)	0.529	322.0
	Recessive	87 (72.5)	56 (70.0)	1.13 (0.61–2.11)	0.702	273.1	69 (62.73)	0.57 (0.90–2.73)	0.114	319.9
	Over dominant	68 (56.7)	47 (58.8)	0.92 (0.52–1.63)	0.770	273.1	69 (62.72)	0.78 (0.46–1.32)	0.349	321.5
	Log-additive	120 (60.0)	80 (40.0)	1.05 (0.72–1.53)	0.799	273.1	110 (47.8)	0.80 (0.57–1.12)	0.184	320.6

Abbreviations: CI, Confidence interval; OR, odds ration; AIC, Akaike information criteria; *p*-values were calculated from logistic regression analysis.

3.3. Haplotype Analysis

Identification of multi-locus interactions between SNVs is an important way to study the pathogenesis of complex diseases. The frequency of haplotypes for gene variants located on one chromosome was investigated. A linkage disequilibrium (LD) of the three SNVs, rs2071010 (FOLR1), rs630074 (FOLR1) and rs61886492 (FOLH1), and two GGH gene variants (rs11545078 and rs3758149) was performed using Haploview 4.2 [34]. A strong linkage disequilibrium was found between the GGH rs11545078/rs3758149 variants

(distance 12,964 bp, D′ = 0.961, LOD = 15.03, r^2 = 0.196) and a weak disequilibrium was found between the FOLR1 rs2071010/rs630074 variants (distance 3973bp, D′ = 0.856, LOD = 2.55, r^2 = 0.034). The FOLH1 gene variant rs61886492 is in linkage equilibrium with the FOLR1 variants. A schematic diagram of the LD pattern is shown in Figure 2.

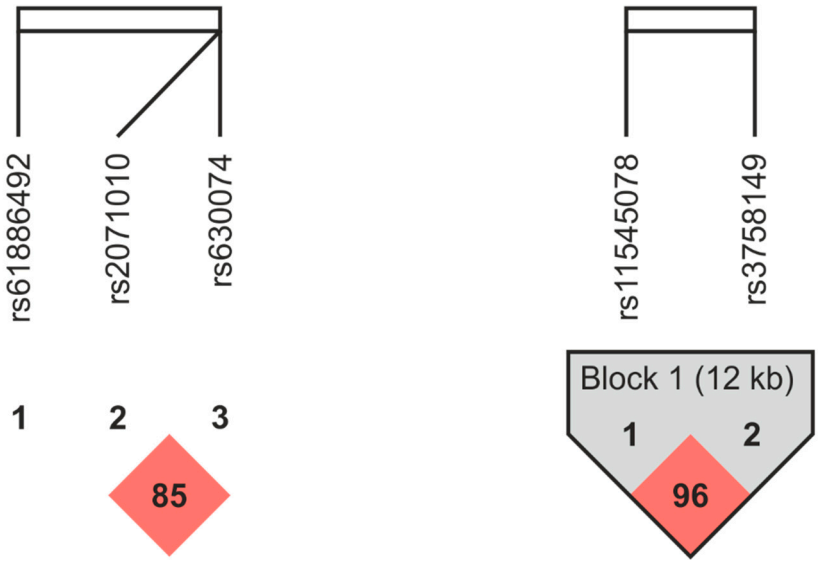


Figure 2. Haploview plot illustrating the linkage disequilibrium of the GGH, FOLR1 and FOLH1 variants in controls and patients. Red squares indicate pairs in LD and numbers indicate the D′ value expressed as a percentile.

Three haplotypes were generated for each of two selected SNVs of the GGH and FOLR1 genes among women diagnosed with fetal death and women from the control group. There were no statistically significant differences in the frequency of haplotypes between the study and control groups. When comparing the IUFD and RM groups with controls, no significant differences were observed (Table 6). A more frequent occurrence of the AC haplotype containing minor alleles of the rs2071010 and rs630074 FOLR1 gene was observed in women with RM compared to controls (frequency 0.056 vs. 0.024 in controls, p = 0.067).

Table 6. The frequency of GGH and FOLR1 haplotypes.

Gene/SNVs	Haplotypes	Frequency (Overall)	Frequency (Case/Ctrl)	Case/Ctrl *p*-Value	IUFD/Ctrl *p*-Value	RM/Ctrl *p*-Value
GGH rs11545078/rs3758149	CC	0.729	0.734/0.720	0.711	0.710	0.792
	CT	0.199	0.200/0.196	0.904	0.832	0.724
	TT	0.071	0.063/0.083	0.344	0.597	0.314
FOLR1 rs2071010/rs630074	GT	0.510	0.485/0.549	0.124	0.184	0.198
	GC	0.448	0.465/0.422	0.299	0.242	0.515
	AC	0.039	0.048/0.024	0.119	0.418	0.067

3.4. Relationship Between Placental and Newborns' Weight with Genotypes

The statistical significance of genotypic effects, along with the mean weights of newborns and placentas in controls, are presented in Table 7. The analysis did not detect any significant associations between the maternal genotypes and the newborns' birth weight or placental weight in any of the analyzed genetic models. Also, after adjusting the model for mother's age, BMI, week of pregnancy and sex of the newborn, no statistically significant differences were obtained.

Table 7. Associations between newborns' weight, placenta weight and genotypes in healthy controls.

SNV	Genotypes			Log-Additive Model	
	0	1	2	*p* Crude	*p* Adj.
		Newborn Weight			
rs2071010	3477.61 ± 385.84	3582.86 ± 616.03	—	0.502	0.448
rs630074	3435.28 ± 363.63	3505.41 ± 428.86	3502.17 ±383.76	0.477	0.599
rs61886492	3479.19 ± 397.77	3540.00 ± 445.62	—	0.663	0.725
rs11545078	3495.90 ± 408.83	3423.00 ± 354.57	—	0.459	0.171
rs3758149	3500.85 ± 429.77	3468.00 ± 374.84	3460.00 ± 371.38	0.657	0.741
rs1051266	3510.61 ± 413.02	3496.54 ± 412.60	3439.43 ± 375.03	0.462	0.284
		Placenta weight			
rs2071010	630.75 ± 114.42	605.71 ± 69.25	—	0.569	0.612
rs630074	612.78 ± 118.87	638.44 ± 112.94	630.87 ± 100.99	0.461	0.410
rs61886492	631.22 ± 114.12	605.56 ± 87.48	—	0.512	0.600
rs11545078	630.75 ± 116.08	622.00 ± 92.88	—	0.752	0.883
rs3758149	619.07 ± 118.09	644.00 ± 106.95	595.00 ± 97.52	0.585	0.638
rs1051266	630.91 ± 98.50	641.83 ± 123.88	609.14 ± 106.23	0.415	0.382

Mean ± SD, *p* adjusted by age, BMI, gestational age and newborn sex.

4. Discussion

4.1. Folate Metabolism and Pregnancy Losses

In this study, we evaluated the associations between selected SNVs genes related to folate uptake and distribution and fetal demise in the Polish population. The etiology of pregnancy loss varies depending on gestational age and, therefore, the causes of miscarriages and intrauterine fetal death may be different. However, the entire pregnancy is a continuous process and any changes at the beginning may manifest themselves at the end. Pregnancy is an extremely stressful period in a woman's life, and the loss of pregnancy at any stage is associated with a significant emotional burden for parents [36].

Folic acid metabolism plays a pivotal role in various biochemical processes, including purine synthesis, thymidine monophosphate (dTMP) production, mitochondrial protein translation, and the regeneration of methionine. These processes underpin essential cellular functions such as proliferation, mitochondrial energy production, and epigenetic modifications. Adequate folate circulation is therefore fundamental for numerous physiological and metabolic pathways, particularly during pregnancy. Disruptions in folate metabolism are strongly associated with adverse pregnancy outcomes.

Evidence highlights the beneficial effects of folate on placental development even in the early stages of gestation. For instance, studies have shown that treating trophoblast tissue with folic acid solutions significantly enhances extravillous trophoblast (EVT) invasion, promotes angiogenesis, and stimulates the secretion of matrix metalloproteinases (MMPs) [37]. Folate requirements during pregnancy increase substantially, by approximately five- to tenfold, to support fetal growth and development [38]. Consequently, any impairment in the transfer of folate from the maternal system to the fetus can contribute to pregnancy complications or reproductive failure.

4.2. Folate Transporters SNVs and Pregnancy Failure

There is not much research on folate transporters and pregnancy failure. Laanpere at al. [39] examined folate-metabolizing gene variants and pregnancy outcomes in Estonian women undergoing IVF or ICSI. By genotyping single-nucleotide polymorphisms, they found that heterozygosity for FOLR1 1816 C/delC (rs3833748) and 1841 G>A (rs1540087) was associated with a raised risk of pregnancy loss. The rs2071010 variant of the FOLR1 gene, also examined in our work, in the Estonian population showed a non-significant trend towards a lower percentage of previously failed IVF treatments (84.94 ± 4.84%) in individuals with the GA genotype compared to individuals with the GG major homozygous

genotype (94.00 ± 1.69%). In our study, a more frequently, non-significant A allele was observed in women with RM compared to controls (0.059 vs. 0.029, $p = 0.115$).

Much more research has been conducted on SLC19A1 gene polymorphisms. In the above-mentioned work by Laanpere et al. [39], the rs1051266 variant was associated with a reduced number of previously failed IVF treatments for GA heterozygotes and was more prevalent among fertile controls [39]. In a case-control study (82 RM women and 166 controls) in the Chinese population (Kunshan City), it was observed that combinations of two rare alleles, SLC19A1 80G-SLC19A1 696C (rs1051266 and rs12659), had a lower frequency in RM patients ($p = 0.084$) [40]. A study by Kim et al. [41] examined the associations between folate metabolic pathway gene polymorphisms and unexplained pregnancy loss (UPL) using matched maternal and fetal samples. No significant differences were detected in fetal samples between the UPL group and the control group. However, the frequencies of allelic combinations (G-T-A) for variants (RFC-1 80G>A, MTHFR 677C>T, MTHFD1 1958G>A) were significantly higher in the samples of mothers from the UPL group than in the controls (OR = 3.577, $p = 0.049$) [41]. Two studies analyzed three polymorphic variants (-43T>C, 80G>A, and 696C>T) of the SLC19A1 gene with recurrent miscarriages. The first one found an association between recurrent miscarriages and the combination of genotypes and haplotypes of these variants in the population of South Korean women [15]. In the second study, the rs1131596 (-43T>C) variant of the SLC19A1 gene was significantly associated with a risk of recurrent miscarriage in Iranian women ($p < 0.05$). Haplotype frequencies of rs/rs/rs were also found to be significantly different in patients than controls in this research [16]. In our population, we examined only one of these variants (80G>A) and found no association with either miscarriages or IUFD. Ananth et al. [42] studied the association between the RFC-1 80G>A polymorphism and placental abruption and did not find any significant differences between this variant, plasma folate levels and abruption risk. The authors suspected that this was related to the high prevalence of prenatal multivitamin and folate supplementation in this population (over 80%) [42]. In our study, we did not examine the patients' serum folate levels, assuming that it would be unreliable due to the supplementation of folic acid (mainly levomefolic acid) by all participants in this project. Also, the difference in gestational age between the groups at the time of sample collection resulted in our decision not to analyze folate metabolites in the patients' serum.

In cases of stillbirth, examining the placenta, which comprises distinct circulatory systems from both the mother and fetus, offers valuable insights into potential causes. Placental pathology has been shown to identify the cause of stillbirth in 64.6% of cases, outperforming other methods such as fetal autopsy (42.4%), genetic testing (11.9%), and antiphospholipid antibody testing (11.1%) [43,44]. Man et al. [45] conducted an analysis of placental pathology in intrauterine fetal deaths spanning from 13 to 40 weeks of gestation, attributing 32% of stillbirths to placental abnormalities. Similarly, Thompson et al. [46] reviewed a clinical database containing 1,256 cases of previously unexplained pregnancy losses between 6 and 43 weeks of gestation. Their examination of placental pathology slides revealed dysmorphic chorionic villi, a feature linked to genetic abnormalities, in 86.2% of unexplained miscarriages (757 cases). For unexplained stillbirths, the most frequent placental anomaly was a small placenta, observed in 33.9% of cases (128 cases) [46]. Given that placental abnormalities and low fetal weight are established risk factors for intrauterine fetal death [47], we restricted our investigation of the relationship between placental and neonatal weights and the studied genetic variants in a control group of healthy mothers. However, our findings revealed no association between the genetic variants implicated in folate transport and distribution and neonatal or placental weights, as assessed in both unadjusted and adjusted models.

Folate receptor autoantibodies (FRAbs) can disrupt folate internalization and metabolism by binding to or blocking folate receptors. Experimental studies on pregnant rats have shown that FRαAb accumulates rapidly in the placenta, impeding folate transport to the fetus. In such cases, higher concentrations of folate were necessary to overcome receptor blockades and restore transport efficiency [48]. These findings suggest that FRαAb could play a significant role in

pregnancy complications, warranting careful evaluation when designing therapeutic strategies involving folic acid supplementation for such conditions [49].

4.3. Glutamate Carboxypeptidase II (FOLH1) Gene

Glutamate carboxypeptidase II (FOLH1) gene encodes three distinct splice variants with different functional roles: N-acetylated-α-linked acidic dipeptidase (NAALADase), folyl poly-γ-glutamate carboxypeptidase (FGCP), and prostate-specific membrane antigen (PSMA) [50]. The rs61886492 variant results in an amino acid substitution from histidine to tyrosine, changing a basic amino acid with an imidazole ring to an aromatic amino acid with a polar-charged group. This alteration in amino acid structure (from positive to neutral) modifies the protein's conformation, which in turn affects its binding capacity. Studies have shown that the H475Y polymorphism reduces the enzyme's activity, leading to lower plasma folate concentrations and higher total plasma homocysteine (tHcy) levels [28,51,52]. The role of this variant was analyzed by Divyya et al. [50] in a group of 1561 people with different disease conditions. They observed a protective role of the H475Y variant in cancers [breast cancer; OR = 0.81 (95%CI: 0.55–1.19), prostate cancer: OR = 0.00 (95%CI: 0.00–0.66)], and in autism (OR = 0.47 (95%CI: 0.21–1.03), whereas an inflated risk was observed in CAD OR = 1.69 (95%CI: 1.20–2.37) and miscarriages [maternal OR = 3.26 (95%CI: 2.11–5.04); paternal OR = 1.99 (95%CI: 1.23–3.21)]. In our study, the rare allele A of the rs61886492 FOLH1 variant increased the risk of IUFD 2.8 times compared to the control group, but unlike the previously discussed Divyya study, we did not observe a statistically significant association of this variant with recurrent miscarriages. We hypothesize that the rs61886492 variant of the FOLH1 gene will probably not have a significant impact on fetal development with appropriate folic acid supplementation. However, during rapid tissue growth occurring in the second and third trimester of pregnancy, the resulting reduced folate absorption may be one of the factors leading to intrauterine fetal death. Although several epidemiological studies have examined the impact of the His475Tyr polymorphism, its precise role in folate uptake and metabolism remains uncertain. Navrátil et al. [53] explored the molecular recognition of polyglutaminated folates by GCPII and aimed to understand how the His475Tyr polymorphism influences the protein's structure and function. Their findings showed that the enzymatic activity of recombinant human GCPII with the His475Tyr variant was similar to the wild-type enzyme. Additionally, X-ray crystallography of the protein's three-dimensional structure did not reveal any significant changes caused by the mutation. However, the authors observed subtle differences that did not fully account for the phenotypic effects associated with this polymorphism. They suggest that the His475Tyr variant might influence folate levels through alternative mechanisms, such as altering the transport function of GCPII, its expression levels, or its interactions with other signaling molecules [53].

4.4. Folic Acid in Pregnancy

Until recently, global recommendations advised women planning a pregnancy to take 400 µg of folic acid daily, from preconception through the first trimester. A randomized controlled trial (RCT) demonstrated that continuing supplementation with 400 µg of folic acid per day during the second and third trimesters could enhance maternal and cord blood folate levels, while also preventing the rise in homocysteine concentrations typically observed in late pregnancy [54]. According to recommendations from the Polish Society of Gynecologists and Obstetricians (PTGiP) for 2024, 800 µg (0.8 mg) of methylated folic acid per day is advised for pregnant and lactating women [55]. Current guidelines suggest a daily folic acid intake of 400 µg before pregnancy, 600 µg during pregnancy, and 500 µg during lactation [56]. While the importance of folic acid during pregnancy is well established, the potential adverse effects of excessive supplementation on the offspring must also be considered [57]. A deeper understanding of the molecular mechanisms that regulate folic acid metabolism will help refine recommendations for appropriate supplementation throughout different stages of pregnancy.

4.5. Strengths and Limitations

Several limitations of our study require caution in interpreting the results. The common polymorphic variants of genes related to folate absorption and distribution that we have selected are just some of many. Other variants of the folic acid metabolism pathway may also be associated with fetal losses. We did not take into account the level of folate or homocysteine in the patients' serum and did not check their dietary habits in detail. It would also be advisable to include FRAbs in the study. Moreover, all the women were of the same race and nationality, and due to the low prevalence IUFD, we could not use a large sample size. Although the power of our research is not sufficient, we selected the patients for the study very carefully. The advantage of the study is the inclusion of women with fetal loss at various stages of pregnancy (RM and IUFD) and the comparisons made between them and mothers of at least two healthy children without a history of pregnancy loss.

5. Conclusions

In conclusion, this study represents one of the first efforts to explore the association between genetic variants in genes involved in folate uptake and distribution and the risk of fetal demise in both recurrent miscarriage (RM) and intrauterine fetal death (IUFD). Our findings suggest that the rs61886492 variant of the FOLH1 gene is linked to an increased risk of IUFD. However, given the limited scope of the current research, further studies are needed to validate these results and investigate the underlying molecular mechanisms. The role of genetic factors is often complex and subtle, making it challenging to interpret their impact, particularly in multifactorial diseases. A deeper understanding of the pathological mechanisms contributing to fetal loss could enhance our knowledge of the interaction between genetic predispositions and environmental factors, ultimately guiding more effective treatments and reducing the risk of pregnancy failure.

Author Contributions: Conceptualization—M.K.-W.; methodology—G.K. and M.K.-W.; software—H.W. and P.O.; validation—T.M.K. and G.K.; formal analysis—H.W. and P.O.; investigation—G.K. and A.E.M.; resources—H.W., P.O. and T.M.K.; material collection—M.K.-W. and A.E.M.; writing—original draft preparation—M.K.-W., A.E.M., B.C. and M.O.; writing—review and editing—M.O. and T.M.K.; visualization—G.K., A.E.M. and M.K.-W.; supervision—H.W. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Local Bioethical Committee at Poznan University of Medical Sciences (Decision No. No 596/19).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the patients to publish this paper.

Data Availability Statement: Data available on request due to restrictions.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Quenby, S.; Gallos, I.D.; Dhillon-Smith, R.K.; Podesek, M.; Stephenson, M.D.; Fisher, J.; Brosens, J.J.; Brewin, J.; Ramhorst, R.; Lucas, E.S.; et al. Miscarriage matters: The epidemiological, physical, psychological, and economic costs of early pregnancy loss. *Lancet* **2021**, *397*, 1658–1667. [CrossRef]
2. Youssef, A.; Lashley, L.; Dieben, S.; Verburg, H.; van der Hoorn, M.L. Defining recurrent pregnancy loss: Associated factors and prognosis in couples with two versus three or more pregnancy losses. *Reprod. Biomed. Online* **2020**, *41*, 679–685. [CrossRef]
3. ESHRE Guideline Group on RPL; Bender Atik, R.; Christiansen, O.B.; Elson, J.; Kolte, A.M.; Lewis, S.; Middeldorp, S.; Mcheik, S.; Peramo, B.; Quenby, S.; et al. ESHRE guideline: Recurrent pregnancy loss: An update in 2022. *Hum. Reprod. Open* **2023**, *2023*, hoad002. [CrossRef] [PubMed]
4. Lawn, J.E.; Blencowe, H.; Waiswa, P.; Amouzou, A.; Mathers, C.; Hogan, D.; Flenady, V.; Frøen, J.F.; Qureshi, Z.U.; Calderwood, C.; et al. Lancet ending preventable stillbirths series study group; Lancet Stillbirth Epidemiology investigator group. Stillbirths: Rates, risk factors, and acceleration towards 2030. *Lancet* **2016**, *387*, 587–603. [CrossRef] [PubMed]

5. Fowler, J.R.; Mahdy, H.; Jack, B.W. Pregnancy. In *StatPearls [Internet]*; StatPearls Publishing: Treasure Island, FL, USA, 2023.
6. Miraglia, N.; Dehay, E. Folate Supplementation in Fertility and Pregnancy: The Advantages of (6S)5-Methyltetrahydrofolate. *Altern. Ther. Health Med.* **2022**, *28*, 12–17. [PubMed]
7. Menezo, Y.; Elder, K.; Clement, A.; Clement, P. Folic acid, folinic acid, 5 methyl tetrahydrofolate supplementation for mutations that affect epigenesis through the folate and one-carbon cycles. *Biomolecules* **2022**, *12*, 197. [CrossRef] [PubMed] [PubMed Central]
8. Radziejewska, A.; Chmurzynska, A. Folate and choline absorption and uptake: Their role in fetal development. *Biochimie* **2019**, *158*, 10–19. [CrossRef]
9. Findley, T.O.; Tenpenny, J.C.; O'Byrne, M.R.; Morrison, A.C.; Hixson, J.E.; Northrup, H.; Au, K.S. Mutations in folate transporter genes and risk for human myelomeningocele. *Am. J. Med. Genet. A* **2017**, *173*, 2973–2984. [CrossRef]
10. *NCBI (2023) SLC19A1 Gene [Internet]*; National Library of Medicine (US), National Center for Biotechnology Information: Bethesda, MD, USA, 2004. Available online: https://www.ncbi.nlm.nih.gov/gene/6573 (accessed on 14 June 2023).
11. Pizzagalli, M.D.; Bensimon, A.; Superti-Furga, G. A guide to plasma membrane solute carrier proteins. *FEBS J.* **2021**, *288*, 2784–2835. [CrossRef]
12. Wang, X.; Wei, H.; Tian, Y.; Wu, Y.; Luo, L. Genetic variation in folate metabolism is associated with the risk of conotruncal heart defects in a Chinese population. *BMC Pediatr.* **2018**, *18*, 287. [CrossRef]
13. Yi, K.; Ma, Y.H.; Wang, W.; Zhang, X.; Gao, J.; He, S.E.; Xu, X.M.; Ji, M.; Guo, W.F.; You, T. The roles of reduced folate carrier-1 (RFC1) A80G (rs1051266) polymorphism in congenital heart disease: A meta-analysis. *Med. Sci. Monit.* **2021**, *27*, e929911-1–e929911-12. [CrossRef]
14. Chatzikyriakidou, A.; Georgiou, I.; Voulgari, P.V.; Papadopoulos, C.G.; Tzavaras, T.; Drosos, A.A. Transcription regulatory polymorphism -43T>C in the 5′-flanking region of SLC19A1 gene could affect rheumatoid arthritis patient response to methotrexate therapy. *Rheumatol. Int.* **2007**, *27*, 1057–1061. [CrossRef] [PubMed]
15. Rah, H.; Choi, Y.S.; Jeon, Y.J.; Choi, Y.; Cha, S.H.; Choi, D.H.; Ko, J.J.; Shim, S.H.; Kim, N.K. Solute Carrier Family 19, member 1 (SLC19A1) polymorphisms (-43T>C, 80G>A, and 696C>T), and haplotypes in idiopathic recurrent spontaneous abortion in a Korean population. *Reprod. Sci.* **2012**, *19*, 513–519. [CrossRef] [PubMed]
16. Mohtaram, S.; Sheikhha, M.H.; Honarvar, N.; Sazegari, A.; Maraghechi, N.; Feizollahi, Z.; Ghasemi, N. An association study of the SLC19A1 gene polymorphisms/haplotypes with idiopathic recurrent pregnancy loss in an Iranian population. *Genet. Test. Mol. Biomark.* **2016**, *20*, 235–240. [CrossRef]
17. Mai, J.; Wu, L.; Yang, L.; Sun, T.; Liu, X.; Yin, R.; Jiang, Y.; Li, J.; Li, Q. Therapeutic strategies targeting folate receptor α for ovarian cancer. *Front. Immunol.* **2023**, *14*, 1254532. [CrossRef]
18. Varaganti, P.; Buddolla, V.; Lakshmi, B.A.; Kim, Y.J. Recent advances in using folate receptor 1 (FOLR1) for cancer diagnosis and treatment, with an emphasis on cancers that affect women. *Life Sci.* **2023**, *326*, 121802. [CrossRef] [PubMed]
19. Marchetti, C.; Palaia, I.; Giorgini, M.; De Medici, C.; Iadarola, R.; Vertechy, L.; Domenici, L.; Di Donato, V.; Tomao, F.; Muzii, L.; et al. Targeted drug delivery via folate receptors in recurrent ovarian cancer: A review. *Onco Targets Ther.* **2014**, *7*, 1223–1236. [CrossRef] [PubMed]
20. Piedrahita, J.A.; Oetama, B.; Bennett, G.D.; van Waes, J.; Kamen, B.A.; Richardson, J.; Lacey, S.W.; Anderson, R.G.; Finnell, R.H. Mice lacking the folic acid-binding protein Folbp1 are defective in early embryonic development. *Nat. Genet.* **1999**, *23*, 228–232. [CrossRef] [PubMed]
21. Elwood, P.C.; Nachmanoff, K.; Saikawa, Y.; Page, S.T.; Pacheco, P.; Roberts, S.; Chung, K.N. The divergent 5′ termini of the alpha human folate receptor (hFR) mRNAs originate from two tissue-specific promoters and alternative splicing: Characterization of the alpha hFR gene structure. *Biochemistry* **1997**, *36*, 1467–1478. [CrossRef] [PubMed]
22. Kim, S.E. Enzymes involved in folate metabolism and its implication for cancer treatment. *Nutr. Res. Pract.* **2020**, *14*, 95–101. [CrossRef]
23. Wang, S.M.; Li, M.; Wu, W.S.; Sun, L.L.; Yan, D. The role of transcription factor Sp1 in the regulation of gamma-glutamyl hydrolase gene expression by the rs3758149 polymorphism in CEM/C1 cells. *Pharmazie* **2019**, *74*, 671–674. [CrossRef] [PubMed]
24. The National Centre for Biotechnology Information (NCBI): Single Nucleotide Polymorphism Database (dbSNP). Available online: https://academic.oup.com/nar/article/28/1/352/2384425 (accessed on 9 January 2024).
25. ClinVar Genomic Variation as It Relates to Human Health. Available online: https://www.ncbi.nlm.nih.gov/clinvar/variation/809757/ (accessed on 9 January 2024).
26. PredictSNV v.2. Available online: https://loschmidt.chemi.muni.cz/predictsnp2/ (accessed on 15 May 2023).
27. Bendl, J.; Musil, M.; Stourac, J.; Zendulka, J.; Damborsky, J.; Brezovsky, J. PredictSNV2: A unified platform for accurately evaluating SNV effects by exploiting the different characteristics of variants in distinct genomic regions. *PLOS Comput. Biol.* **2016**, *12*, e1004962. [CrossRef] [PubMed]
28. Devlin, A.M.; Ling, E.H.; Peerson, J.M.; Fernando, S.; Clarke, R.; Smith, A.D.; Halsted, C.H. Glutamate carboxypeptidase II: A polymorphism associated with lower levels of serum folate and hyperhomocysteinemia. *Hum. Mol. Genet.* **2000**, *9*, 2837–2844. [CrossRef]
29. Hayashi, H.; Fujimaki, C.; Inoue, K.; Suzuki, T.; Itoh, K. Genetic polymorphism of C452T (T127I) in human gamma-glutamyl hydrolase in a Japanese population. *Biol. Pharm. Bull.* **2007**, *30*, 839–841. [CrossRef]

30. Dervieux, T.; Kremer, J.; Lein, D.O.; Capps, R.; Barham, R.; Meyer, G.; Smith, K.; Caldwell, J.; Furst, D.E. Contribution of common polymorphisms in reduced folate carrier and gamma-glutamylhydrolase to methotrexate polyglutamate levels in patients with rheumatoid arthritis. *Pharmacogenetics* **2004**, *14*, 733–739. [CrossRef]
31. Untergasser, A.; Cutcutache, I.; Koressaar, T.; Ye, J.; Faircloth, B.C.; Remm, M.; Rozen, S.G. Primer3—New capabilities and interfaces. *Nucleic Acids Res.* **2012**, *40*, e115. [CrossRef] [PubMed]
32. R Core Team. *R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2023. Available online: https://www.R-project.org (accessed on 1 February 2024).
33. Moreno, V.; Gonzalez, J.; Pelegri, D. SNPassoc: SNPs-Based Whole Genome Association Studies. R package version 2.1-0. 2022. Available online: https://CRAN.R-project.org/package=SNPassoc (accessed on 15 May 2024).
34. Haplovie w 4.2. Available online: http://www.broad.mit.edu/mpg/haploview/ (accessed on 9 June 2024).
35. Moore, C.; Jacobson, S. Genpwr: Power Calculations Under Genetic Model Misspecification. R Package Version 1.0.4. 2021. Available online: https://CRAN.R-project.org/package=genpwr (accessed on 9 June 2024).
36. Donegan, G.; Noonan, M.; Bradshaw, C. Parents experiences of pregnancy following perinatal loss: An integrative review. *Midwifery* **2023**, *121*, 103673. [CrossRef]
37. Williams, P.J.; Bulmer, J.N.; Innes, B.A.; Pipkin, F.B. Possible roles for folic acid in the regulation of trophoblast invasion and placental development in normal early human pregnancy. *Biol. Reprod.* **2011**, *84*, 1148–1153. [CrossRef]
38. Antony, A.C. In utero physiology: Role of folic acid in nutrient delivery and fetal development. *Am. J. Clin. Nutr.* **2007**, *85*, 598S–603S. [CrossRef]
39. Laanpere, M.; Altmäe, S.; Kaart, T.; Stavreus-Evers, A.; Nilsson, T.K.; Salumets, A. Folate-metabolizing gene variants and pregnancy outcome of IVF. *Reprod. Biomed. Online* **2011**, *22*, 603–614. [CrossRef]
40. Cao, Y.; Zhang, Z.; Zheng, Y.; Yuan, W.; Wang, J.; Liang, H.; Chen, J.; Du, J.; Shen, Y. The association of idiopathic recurrent early pregnancy loss with polymorphisms in folic acid metabolism-related genes. *Genes Nutr.* **2014**, *9*, 402. [CrossRef] [PubMed]
41. Kim, J.Y.; Kim, J.W.; Sung, S.R.; Park, J.E.; Shim, S.H.; Cha, D.H. Impact of RFC1, MTHFR, and MTHFD1 polymorphism on unexplained pregnancy loss (UPL): Comparative analysis of maternal and fetal components using mother-abortus paired samples. *Eur. J. Obstet. Gynecol. Reprod. Biol.* **2018**, *231*, 152–157. [CrossRef]
42. Ananth, C.V.; Peltier, M.R.; Moore, D.F.; Kinzler, W.L.; Leclerc, D.; Rozen, R.R. New Jersey-Placental Abruption Study Investigators. Reduced folate carrier 80A-->G polymorphism, plasma folate, and risk of placental abruption. *Hum. Genet.* **2008**, *124*, 137–145. [CrossRef]
43. Management of Stillbirth: Obstetric Care Consensus No, 10. *Obstet. Gynecol.* **2020**, *135*, e110–e132, Erratum in *Obstet. Gynecol.* **2023**, *141*, 1030. [CrossRef] [PubMed]
44. Waller, J.A.; Saade, G. Stillbirth and the placenta. *Semin. Perinatol.* **2024**, *48*, 151871. [CrossRef] [PubMed]
45. Man, J.; Hutchinson, J.C.; Heazell, A.E.; Ashworth, M.; Jeffrey, I.; Sebire, N.J. Stillbirth and intrauterine fetal death: Role of routine histopathological placental findings to determine cause of death. *Ultrasound Obstet. Gynecol.* **2016**, *48*, 579–584. [CrossRef] [PubMed]
46. Thompson, B.B.; Holzer, P.H.; Kliman, H.J. Placental pathology findings in unexplained pregnancy losses. *Reprod. Sci.* **2024**, *31*, 488–504. [CrossRef]
47. Silver, R.M.; Reddy, U. Stillbirth: We can do better. *Am. J. Obstet. Gynecol.* **2024**, *231*, 152–165. [CrossRef]
48. Bobrowski-Khoury, N.; Sequeira, J.M.; Arning, E.; Bottiglieri, T.; Quadros, E.V. Absorption and tissue distribution of folate forms in rats: Indications for specific folate form supplementation during pregnancy. *Nutrients* **2022**, *14*, 2397. [CrossRef]
49. Qin, X.Y.; Ha, S.Y.; Chen, L.; Zhang, T.; Li, M.Q. Recent advances in folates and autoantibodies against folate receptors in early pregnancy and miscarriage. *Nutrients* **2023**, *15*, 4882. [CrossRef] [PubMed]
50. Divyya, S.; Naushad, S.M.; Addlagatta, A.; Murthy, P.V.; Reddy, C.R.; Digumarti, R.R.; Gottumukkala, S.R.; Kumar, A.; Rammurti, S.; Kutala, V.K. Paradoxical role of C1561T glutamate carboxypeptidase II (GCPII) genetic polymorphism in altering disease susceptibility. *Gene* **2012**, *497*, 273–279. [CrossRef]
51. Halsted, C.H.; Wong, D.H.; Peerson, J.M.; Warden, C.H.; Refsum, H.; Smith, A.D.; Nygård, O.K.; Ueland, P.M.; Vollset, S.E.; Tell, G.S. Relations of glutamate carboxypeptidase II (GCPII) polymorphisms to folate and homocysteine concentrations and to scores of cognition, anxiety, and depression in a homogeneous Norwegian population: The Hordaland Homocysteine Study. *Am. J. Clin. Nutr.* **2007**, *86*, 514–521. [CrossRef] [PubMed]
52. Singh, B.; Gupta, R.; Rai, S.K.; Rai, S.; Singh, R. In-silico study of neural tube defect in relation to FOHL1 gene. *Int. J. Sci. Res.* **2014**, *3*, 366–367. Available online: https://www.worldwidejournals.com/international-journal-of-scientific-research-(IJSR)/fileview.php?val=April_2014_1397472981_7b339_130.pdf (accessed on 5 February 2024).
53. Navrátil, M.; Ptáček, J.; Šácha, P.; Starková, J.; Lubkowski, J.; Bařinka, C.; Konvalinka, J. Structural and biochemical characterization of the folyl-poly-γ-l-glutamate hydrolyzing activity of human glutamate carboxypeptidase II. *FEBS J.* **2014**, *281*, 3228–3242. [CrossRef] [PubMed]
54. McNulty, B.; McNulty, H.; Marshall, B.; Ward, M.; Molloy, A.M.; Scott, J.M.; Dornan, J.; Pentieva, K. Impact of continuing folic acid after the first trimester of pregnancy: Findings of a randomized trial of folic acid supplementation in the second and third trimesters. *Am. J. Clin. Nutr.* **2013**, *98*, 92–98. [CrossRef] [PubMed]

55. Seremak-Mrozikiewicz, A.; Bomba-Opoń, D.; Drews, K.; Kaczmarek, P.; Wielgoś, M.; Sieroszewski, P. Stanowisko Ekspertów Polskiego Towarzystwa Ginekologów i Położników w zakresie suplementacji aktywnych folianów, choliny i witamin B6 i B12 w okresie przedkoncepcyjnym, ciąży i połogu. *Ginekol. Perinatol. Prakt.* **2024**, *9*, 154–156.
56. Stamm, R.A.; Houghton, L.A. Nutrient intake values for folate during pregnancy and lactation vary widely around the world. *Nutrients* **2013**, *5*, 3920–3947. [CrossRef] [PubMed]
57. Xu, X.; Zhang, Z.; Lin, Y.; Xie, H. Risk of excess maternal folic acid supplementation in offspring. *Nutrients* **2024**, *16*, 755. [CrossRef] [PubMed]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.