

# THE ROLE OF POLYMORPHISMS IN GENES OF FOLATE METABOLISM AND HYPERHOMOCYSTEINEMIA IN REALIZATION OF MISSED ABORTION IN THE 1ST TRIMESTER

The article explores mechanisms of non-developing the 1st trimester pregnancy on the basis of studying frequency of polymorphic alleles in folate metabolism genes MTHFR C677T, MTHFR A1298C, MTRR A66G, MTR A2756G, homocysteine level, platelet and plasma haemostasis sections.

Polymorphism in MTHFR, MTRR, and MTR genes of folate metabolism causes hyperhomocysteinemia and thrombophilic changes. In conditions of genetically accustomed thrombophilic changes the desynchronization of fibrinolysis and fibrin formation processes during implantation occurs that leads to poor trophoblast invasion and its inadequacy, which in turn causes miscarriage due to non-developing pregnancy in the I trimester.

**Keywords:** Missing abortion, folate cycle, MTHFR, MTRR, MTR, hyperhomocysteinemia, thrombophilia.

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NAGIMA MAMEDALIEVA,  
ALIJA AIMBETOVA,  
GULNARA SVYATOVA

*Scientific Center of Obstetrics,  
Gynecology and Perinatology  
Kazakhstan*

## Introduction

One of the major places in the structure of pregnancy loss, especially in regard to its early miscarriages, is taken by non-developing pregnancy - i.e. pre-natal death of the embryo without spontaneous expulsion from the uterine cavity and with retention of gestational sac for an indefinite period of time (WHO criteria, 1980). The ratio of non-developing pregnancies reaches 15% among spontaneous abortions and up to 45-80% in the early pregnancy. Kulakovskiy et al. (2007) noted that retention of the dead gestational sac in the womb affects subsequent reproductive function of the woman, threatens her life and health including haemostasiological complications expressed in varying degrees.

It is known that etiological structure of recurrent miscarriage has genetic, anatomical, infectious and immunological factors. However, according to Ilizarova et al. (2008), the cause of recurrent miscarriage in 20-40% of cases is not completely clear. Genetic defects deserve particular attention. According to Radzinskiy (2007), failure of utero-feto-placental blood flow is one of pathogenetic mechanisms which cause non-developing pregnancy development. Studying of genetic polymorphisms which cause certain changes in the process of blood clotting is of great interest. In 50% of cases, recurrent miscarriage is the result of hereditary and acquired thrombophilia which includes hyperhomocysteinemia (HHC).

There are genetically determined and acquired causes for impaired homocysteine (HC) metabolism. According to Fillipova (2008) and Makatsaria (2007), the first group includes mutations in genes of such important enzymes as cystathionine- $\beta$ -synthetase (CBS) and methylenetetrahydrofolate reductase (MTHFR) which lead to deficiency of enzymes responsible for metabolism of this acid. There are homozygous and heterozygous forms of genetic defects.

Development mechanisms of vascular complications of hyperhomocysteinemia are currently being intensively studied. Rodgers (1986), Ozolinya et al. (2003) suggest that hyperhomocysteinemia affects a number of mechanisms involved in thrombogenesis including coagulation cascade, vessel-thrombocytic section, oxidation-reduction reactions, endothelium, and vascular smooth muscle cells.

HC metabolism is carried out in folate cycle which involves a large number of enzymes requiring sufficient amounts of folic acid and vitamin B complex for successful activity. The key enzyme of folate cycle is MTHFR which converts folic acid into its active form, 5-methyltetrahydrofolate. There is a number of allelic variants of this gene causing severe enzyme deficiency, but most of these variants are rare. Two polymorphisms, C677T and A1298C, are of practical importance. MTRR enzyme (methionine synthase reductase) is involved in restoration of MTR (methionine synthase) activity, the enzyme directly involved in homocysteine methylation. According to Fetisova (2007), polymorphic variants of MTHFR and MTRR genes may be regarded as a risk factor for development of certain diseases. However, their role in the etiopathogenesis of various diseases has not been determined. Since all the enzymes are involved in one cascade, researchers discuss the possibility of intergenic interactions. Combination of polymorphic alleles in various genes can significantly influence development of a particular disease and exacerbate its clinical course.

Prediction of non-developing pregnancy will contribute to timely development and implementation of all basic measures to prevent pregnancy complications in women of reproductive age.

Research objective was to understand development mechanisms of non-developing I trimester pregnancy on the basis of studying frequency of polymorphic alleles in folate metabolism genes MTHFR C677T, MTHFR A1298C, MTRR A66G, MTR A2756G, homocysteine level, platelet and plasma haemostasis sections.

## Material and methods

To determine the frequency of alleles and genotypes in folate metabolism genes, a molecular genetic testing has been conducted in 207 women of Kazakh population. The index group included 102 patients with non-developing pregnancy history. The control group included 105 women with normal reproductive function.

Samples of dark blood were taken from the patients and placed into vacutainers with EDTA. DNA was extracted by semi-automated system 6100 Nucleic Acid PrepStation ABI PRISM for extraction of DNA/RNA. Then allele-specific PCR with RFLP for detection of gene polymorphisms was carried out. PCR was performed with the use of Eppendorf PCR amplifier.

In order to determine HC level and parameters of platelet and plasma haemostasis sections, patients of miscarriage risk group were examined by type of non-developing pregnancy both out of pregnancy and in the I trimester, which comprised the index group, and women with no reproductive miscarriages (control group). Homocysteine investigation was carried out by immunoenzyme method.

Statistical analysis included calculation of averages and their errors. Reliability of differences was assessed using Student's t-test.  $\chi^2$  criteria were used to compare distribution of genotypes between the groups.

## Results and discussion

See Table 1 for genotype frequencies in the studied polymorphisms of MTHFR, MTRR, MTR genes in patients with non-developing pregnancy history, as well as in patients with normal reproductive function in the control group.

As presented in Table 1, analysis of C677T MTHFR polymorphism frequencies showed that favorable homozygous genotype (CC) was significantly less frequent in the index group ( $26.5 \pm 4.4\%$ ), while it came up to  $42.9 \pm 4.8\%$  ( $\chi^2 = 6.12$ ;  $p < 0.05$ ) in the control group. Frequency of homozygotes in the T allele (TT) was  $29.4 \pm 4.5\%$  in the index group which exceeded that of the control group -  $4.8 \pm 2.1\%$  ( $\chi^2 = 22.4$ ;  $p < 0.05$ ). Frequency of heterozygotes (CT) in the studied groups had no significant differences.

TABLE 1. GENOTYPES OF FOLATE METHABOLISM GENES

Genotypes	Index group (n=102)		Control group (n=105)		$\chi^2$
	n	% $\pm$ m	n	% $\pm$ m	
C677 allele of MTHFR gene					
CC	27	26.5 $\pm$ 4.4*	45	42.9 $\pm$ 4.8	6.12*
CT	45	44.1 $\pm$ 4.9	55	52.4 $\pm$ 4.9	
TT	30	29.4 $\pm$ 4.5*	5	4.8 $\pm$ 2.1	22.4*
Total	102	100.0	105	100.0	-
A1298C allele of MTHFR gene					
AA	30	29.4 $\pm$ 4.5*	50	47.6 $\pm$ 4.9	7.32*
AC	51	50.0 $\pm$ 5.0	45	42.9 $\pm$ 4.8	
CC	21	20.6 $\pm$ 4.0*	10	9.5 $\pm$ 2.9	4.97*
Total	102	100.0	105	100.0	
A66G allele of MTRR gene					
AA	47	46.1 $\pm$ 4.9*	67	63.8 $\pm$ 4.7	6.57*
AG	36	35.3 $\pm$ 4.7	32	30.5 $\pm$ 4.5	
GG	19	18.6 $\pm$ 3.9*	6	5.7 $\pm$ 2.3	8.12*
A2756G allele of MTR gene					
AA	36	35.3 $\pm$ 4.7*	69	65.7 $\pm$ 4.6	19.16*
AG	49	48.0 $\pm$ 4.9*	31	29.5 $\pm$ 4.5	7.48*
GG	17	16.7 $\pm$ 3.7*	5	4.8 $\pm$ 2.1	7.72*

Note:\* - have significant differences from control group datas ( $p < 0.05$ ).

When studying A1298C polymorphism frequencies, it was noted that favorable genotype (AA) was significantly less frequent in the index group (29.4 $\pm$ 4.5%), whereas it came up to 47.6 $\pm$ 4.9% ( $\chi^2 = 7.23$ ;  $p < 0.05$ ) in the control group. Frequency of homozygotes in the C allele (CC) of the index group was 20.6 $\pm$ 4.0% which was 2 times higher than that of the control group - 9.5 $\pm$ 2.9% ( $\chi^2 = 22.4$ ;  $p < 0.05$ ). Frequency of heterozygotes (CT) in the group of patients with a history of miscarriages uncertainly exceeds that of the population control group (50 $\pm$ 5% and 42.9 $\pm$ 4.8% respectively).

A66G polymorphism analysis of MTRR gene showed that favorable homozygous genotype (AA) was significantly less frequent in the index group (46.1 $\pm$ 4.9%) while in the control group it came up to 63.8 $\pm$ 4.7 ( $\chi^2 = 6.57$ ;  $p < 0.05$ ). Frequency of homozygotes in the G allele (GG) was 18.6 $\pm$ 3.9% in the index group which was more than 3 times higher than that of healthy women - 5.7 $\pm$ 2.3% ( $\chi^2 = 8.12$ ;  $p < 0.05$ ). Frequency of heterozygotes (AG) in the studied groups had no significant differences.

When analyzing genotype frequencies by A2756G polymorphism of MTR gene, it was found that favorable genotype (AA) was significantly less frequent in the index group (35.3 $\pm$ 4.7%) while it came up to 65.7 $\pm$ 4.6% ( $\chi^2 = 19.16$ ,  $p < 0.05$ ) in the control group. Frequency of homozygotes in the G allele (GG) was 16.7 $\pm$ 3.7% in the index group which was more than 3 times higher than that of the control group - 4.8 $\pm$ 2.1% ( $\chi^2 = 7.72$ ;  $p < 0.05$ ). Frequency of heterozygotes (AG) in the group of patients examined with respect to non-developing pregnancy development came up to 48.0 $\pm$ 4.9% which significantly exceeded that of the control group - 29.5 $\pm$ 4.5% ( $\chi^2 = 7.48$ ;  $p < 0.05$ ).

The results of our further investigations showed that HC levels in the blood serum of patients in the risk group of non-developing pregnancy development exceeded the value out of pregnancy in the control group, but there were no significant differences: 13.56 $\pm$ 1.23  $\mu\text{mol/l}$  vs. 11.5 $\pm$ 0.54  $\mu\text{mol/l}$ .

Determination of this value in pregnancy showed that pregnant women who are at high risk for non-developing pregnancy development, HC level in the I trimester increased significantly and came up to 18.25 $\pm$ 1.76  $\mu\text{mol/l}$ , which was significantly higher than that of the control group - 10.4 $\pm$ 0.8  $\mu\text{mol/l}$  ( $p < 0.001$ ). This suggests that lack of HC level reduction in the blood serum during the I trimester of pregnancy is one of pathogenetic reasons for miscarriage due to non-developing pregnancy.

These results indicate possible contribution of MTHFR, MTRR, and MTR genes in the etiology and risk of non-developing pregnancy of the first trimester, which is probably conditioned by toxic effects of excessive HC levels, the concentration of which increases due to the low activity of the above enzymes, but is also conditioned by HC effect on the blood coagulation which is one of the major predisposing factors for early reproductive losses.

Prediction of risk for non-developing pregnancy development in patients with mutations in MTHFR, MTRR, and MTR genes cannot be made solely on the basis of genotype determination without regard to their associations and HC level as the latter can be not only a consequence of mutations in folate metabolism genes but also an independent risk factor for thrombosis.

Considering the ability of homocysteine to influence all stages of thrombosis, we analyzed parameters of platelet and plasma haemostasis sections in the group of women at risk of non-developing pregnancy development and women with normal reproductive function out of and during pregnancy.

Our studies of platelet haemostasis section showed that significant changes in platelet haemostasis section were observed in patients of the risk group for miscarriage even out of pregnancy: for example, platelet count in the index group patients was significantly lower than that of the control group ( $P < 0.05$ ), (Table 2).

TABLE 2. PLATELET HAEMOSTASIS SECTION INDICES (OUT OF PREGNANCY)

Indices	Index group (n=30)	Control group (n=30)	P
Platelet quantity ( $1 \times 10^9$ g/l)	$289.2 \pm 8.4$	$317 \pm 12.1$	$P < 0.05$
Platelet aggregation with ADP (%)	$76.3 \pm 5.9$	$58.5 \pm 4.9$	$P < 0.02$
Platelet aggregation with collagen (%)	$94.7 \pm 6.2$	$49.4 \pm 2.7$	$P < 0.001$
Platelet aggregation with adrenaline (%)	$83.7 \pm 4.5$	$62.5 \pm 3.1$	$P < 0.001$

TABLE 3. PLATELET HAEMOSTASIS SECTION INDICES DURING PREGNANCY.

Indices	Index group (n=30)	Control group (n=30)	P
Platelet quantity ( $1 \times 10^9$ g/l)	$286 \pm 10.2$	$304.4 \pm 15.4$	$P > 0.05$
Platelet aggregation with ADP (%)	$75.6 \pm 3.7$	$62.4 \pm 2.8$	$P < 0.01$
Platelet aggregation with collagen (%)	$85.2 \pm 4.1$	$55.1 \pm 3.4$	$P < 0.001$
Platelet aggregation with adrenaline (%)	$88.4 \pm 3.4$	$66.2 \pm 4.1$	$P < 0.001$

The study of functional activity of platelets showed that platelet aggregation with a weak biological stimulator such as adrenalin was significantly higher in the index group compared with that of the control group:  $83.7 \pm 4.5$  and  $62.5 \pm 3.1$  ( $P < 0.001$ ). Further, with the use of potent inducers (ADP and collagen) allowing to assess maximum platelet aggregability, it was found that aggregation with ADP in the index group was 30% higher than that of the control group ( $76.3 \pm 5.9$  and  $58.5 \pm 4.9$  respectively), ( $P < 0.02$ ). The study of platelet aggregation with collagen showed similar data: the value in the index group was  $94.7 \pm 6.2$  which was almost 2 times higher than that of the control group ( $49.4 \pm 2.7$ ), ( $P < 0.001$ ).

When studying the platelet haemostasis section during the I trimester of pregnancy in patients of the risk group for miscarriage due to non-developing pregnancy and in healthy pregnant women, the following changes were revealed (Table 3). Platelet count in the index group was comparable to that of the control group -  $286 \pm 10.2$  and  $304.4 \pm 15.4$  respectively. Aggregation with adrenaline in the index group was 34% above the benchmark ( $88.4 \pm 3.4$  and  $66.2 \pm 4.1$  respectively), ( $P < 0.01$ ), platelet aggregation with ADP in the index group was 21% above the values in the control group ( $75.6 \pm 3.7$  and  $62.4 \pm 2.8$  respectively) ( $P < 0.01$ ), and aggregation with collagen was 55% higher than that of the control group ( $85.2 \pm 4.1$  and  $55.1 \pm 3.4$  respectively), ( $P < 0.001$ ).

As indicated in Table 3, patients in the index group out of and during pregnancy have lower platelet count compared to that of the control group, which does not change after occurrence of pregnancy. Similar changes occur with qualitative indicators of platelet function: platelet aggregation with ADP, collagen and adrenaline after occurrence of pregnancy has no significant changes either in women in the risk group for miscarriage, or in healthy women. We assume that decrease in the number of platelets in the index group indicates the consumption of platelets due to the increased thrombosis.

The study of plasma haemostasis section parameters in patients threatened by miscarriage showed that out of pregnancy, concentration of soluble fibrin monomer complexes which are markers of thrombinemia was significantly higher:  $6.9 \pm 1.2$  and  $3.6 \pm 0.6$  ( $P < 0.01$ ). Thrombin clotting time in patients of the risk group for miscarriage was significantly higher than that of the control group ( $P < 0.05$ ). Comparison to other values of plasma haemostasis section in the index group did not differ from that of the control group.

Analysis of plasma haemostasis section values in the I trimester of pregnancy showed that significant differences, as well as during pregnancy, were obtained only when comparing values of SFMC -  $10.9 \pm 1.8$  and  $7.6 \pm 1.0$  ( $P < 0.01$ ).

When analyzing parameters of plasma haemostasis section (Table 4) taking place in the course of pregnancy, it was found that these values did not change in both groups. The exception was indicator of SFMC quantity: for example, in the index group, its amount has increased in the first trimester of pregnancy compared to values out of pregnancy ( $6.9 \pm 1.2$  and  $10.9 \pm 1.4$  g/l respectively), ( $P < 0.05$ ). In the control group, this value has also significantly increased after occurrence of pregnancy ( $3.6 \pm 0.6$  and  $7.0 \pm 1.2$  g/l respectively).

TABLE 4. PLAZMA HAEMOSTASIS SECTION PARAMETERS DYNAMIC DURING AND OUT OF PREGNANCY

Indices	Index group		Control group	
	Out of preg.	Pregnants	Out of preg.	Pregnants
APTT (sec)	$27 \pm 3.9$	$22.8 \pm 1.8$	$25.7 \pm 2.9$	$25.2 \pm 1.1$
Thrombin clotting time (sec)	$13.4 \pm 0.5$	$13.3 \pm 0.8$	$12.0 \pm 0.4$	$12.1 \pm 0.6$
Prothrombin clotting time (sec)	$16.3 \pm 1.6$	$15.7 \pm 1.7$	$14.5 \pm 2.3$	$17.4 \pm 1.1$
Prothrombin index	$104.6 \pm 10.2$	$110.4 \pm 8.7$	$99.5 \pm 6.1$	$111.3 \pm 6.3$
SFMC (g/l)	$6.9 \pm 1.2^*$	$10.9 \pm 1.4$	$3.6 \pm 0.6^{**}$	$7.0 \pm 1.2$
Fibrinogene concentration (g/l)	$2.5 \pm 0.8$	$3.3 \pm 0.3$	$2.3 \pm 0.5$	$2.7 \pm 0.2$
Factor XIII (fibrin stabilised)	$23.7 \pm 4.2$	$26.4 \pm 3.9$	$25 \pm 0.7$	$25 \pm 1.2$

Note: \*  $P < 0.05$ , \*\*  $P < 0.01$

Thus, the study of haemostatic system in patients of the risk group for non-developing pregnancy development conducted out of and in the Ist trimester of pregnancy showed significant changes in the haemostatic system. These changes generally related to thrombocytic link and some parameters of plasma haemostasis section occur as early as out of pregnancy and do not undergo significant changes after the occurrence of pregnancy. These features of haemostatic system account for pathogenetic mechanism of fetal death in early pregnancy, as thrombophilic disorders may cause implantation defects and reduce the depth of trophoblast invasion.

Thus, polymorphism in MTHFR, MTRR, and MTR genes of folate metabolism causes hyperhomocysteinemia and thrombophilic changes. In terms of genetically conditioned thrombophilic changes, desynchronization of fibrinolysis and fibrin formation processes take place during implantation leading to poor trophoblast invasion and its inadequacy, which in turn cause miscarriage due to non-developing pregnancy in the I trimester.

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