

## THE IMPORTANCE OF MTHFR GENE MUTATION DETECTION IN PATIENT WITH RECURRENT MISCARRIAGES

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Homocysteine is an enzyme encoded by MTHFR (methylenetetrahydrofolate reductase) gene located on chromosome 1. Mutations in MTHFR gene may result in the afflicted metabolism of homocysteine and thus might increase the risk of recurrent miscarriages. In some cases, recurrent pregnancy loss could be prevented by prescribing folic acid and B group vitamin supplements. The demand of MTHFR gene sequencing for variations is commonly overlooked by doctors or genetic counsellors. To highlight this problem we present a case study of recurrent miscarriages in a patient with a homozygous c. 655C>T variation in MTHFR gene. Moreover, we discuss the need of molecular genetic testing for MTHFR gene variations in patients with recurrent miscarriages and the treatment of hyperhomocysteinemia.

*Key words:* homocysteine, folic acid, . MTHFR, recurrent miscarriage

### INTRODUCTION

Homocysteine is an amino acid serving as an intermediate in methionine metabolism. It is reported that after auto-oxidation of homocysteine it might form biologically reactive products that are associated with increased cell toxicity. Also, homocysteine has been identified to participate in four essential mechanisms of disease: thrombosis, oxidant stress, apoptosis and cellular proliferation (MARON & LOSCALZO, 2009).

There are three main biochemical pathways for homocysteine metabolism shown in Figure1.

One way is methylenetetrahydrofolate reductase (MTHFR) conversion of 5,10-methylene tetrahydrofolate to 5-methyl tetrahydrofolate and affects the activity of cellular cycles participating in nucleotide synthesis, DNA repair, genome stability, maintenance of methyl pool and gene regulation. Another way is remethylation that can be disturbed by defects in cobalamine (vitamin B<sub>12</sub>) metabolism or the deficiency of MTHFR due to mutations in its genes. Other genetic

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determinants such as factor V Leiden (1691 G>A) and prothrombin (20210 G>A) mutations are also important factors for thrombophilia and recurrent miscarriages (NAGORNI-OBRAĐOVIĆ *et al.*, 2014). Elevated levels of homocysteine and decreased methionine are the results of remethylation defects. Trans-sulphuration is the third pathway. Defect in trans-sulphuration is due to a deficiency of cystathionine  $\beta$ -synthase (CBS) which, with its co-factor vitamin B<sub>6</sub> (pyridoxine), converts homocysteine into cystathionine (BLOM & SMULDERS, 2011). It is known that human CBS is expressed in liver, kidneys, muscle, brain and ovary and also during early embryogenesis in the neural and cardiac systems (MARON & LOSCALZO, 2009). The level of homocysteine is effected by various factors including inflammation, so before the analysis of biochemical parameters in serum it is important to check the level of inflammatory mediators (SERAPINAS *et al.*, 2012).

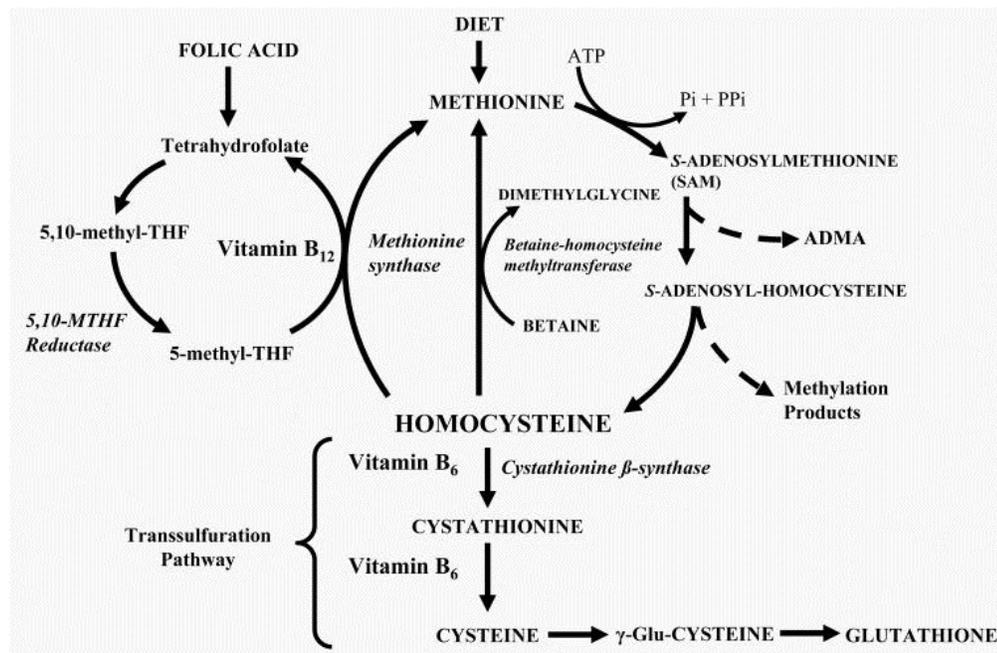


Fig. 1 Metabolic pathways of homocysteine

In this case report we present 30 years old female patient K. V. who was diagnosed with a homozygous mutation in MTHFR gene even though the level of homocysteine in the blood was within the normal range. The patient was referred to clinical geneticist for a consultation because of recurrent miscarriages. In June, 2010 and October, 2013 the patient was diagnosed with not developing pregnancies following first-trimester miscarriages.

**CASE REPORT**

We present 30 years patient, that had two spontaneous abortions. First miscarriage in 2010, second miscarriage in 2013. Both cases were at 6-7 weeks of gestation. The anamnesis showed that working conditions, personal habits or previous health issues may not have contributed to recurrent miscarriages. Genetic counselling was conducted by a clinical geneticist. The genealogy of the family was found not significant in the case. It is shown in Figure 2.

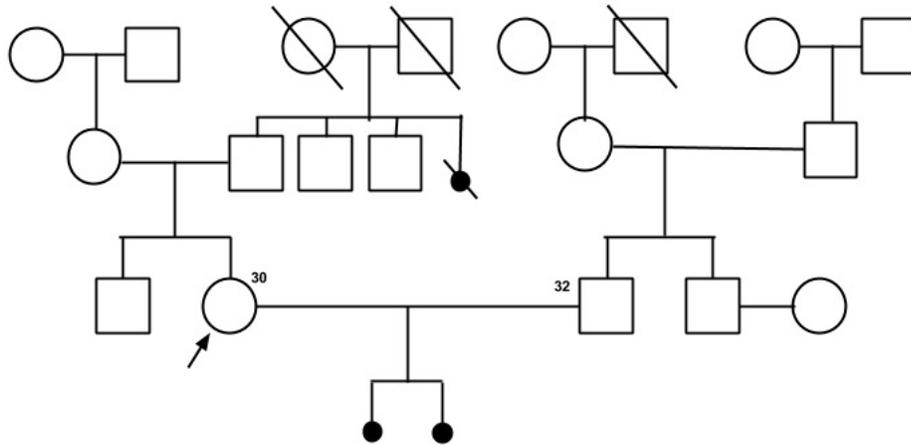


Fig.2 Pedigree of the patient with recurrent miscarriages. The proband is marked with a filled circle

Homocysteine test was performed to determine if the patient has vitamin B12 or folate deficiency as homocysteine in serum is increased markedly in patients with cobalamin or folate deficiency. Homocysteine test result was 11,2 mol/l. Normal ranges of homocysteine in different patients groups (REFSUM *et al*, 2006) as well in pregnant women (WALKER *et al*, 1999) are shown in Table 1

Table 1. Homocysteine level ranges in different groups

Individual group	Homocysteine level
Age < 30 years	4.6 - 8.1 $\mu\text{mol/l}$
Age 30 -59 years	6.3 - 11.2 $\mu\text{mol/l}$
Age > 60 years	5.9-15.3 $\mu\text{mol/l}$
Pregnant 8-16 weeks	3.9-7.3 $\mu\text{mol/l}$
Pregnant 20-28 weeks	3.5-5.3 $\mu\text{mol/l}$
Pregnant 36-42 weeks	3.3-7.5 $\mu\text{mol/l}$
Moderate hyperhomocysteinemia	16-30 $\mu\text{mol/l}$

After results of MTHFR gene analysis the patient received supplementation with B6 vitamin (50mg/d) and B12 vitamin (1mg/d). After one month of treatment the level of homocysteine decreased till 8.4  $\mu\text{mol/l}$  and after two month till 6.4  $\mu\text{mol/l}$ .

Even though result of the test didn't reach level of moderate hyperhomocysteinemia (Table 1), patient's sample was referred to the Centre of Medical Genetics for screening of mutations in MTHFR gene.

Activated partial thromboplastin time (APTT) and Lupus anticoagulant tests were ordered to help to determine the cause of recurrent miscarriage. APTT is used to measure the activity of coagulation pathways. The Lupus anticoagulant as well as APTT is associated with an increased risk of antiphospholipid antibody syndrome (APS), an autoimmune disorder that is involved in blood clot formation and recurrent miscarriages. Lupus anticoagulant is also associated with clot formation in blood vessels of the placenta leading to underdeveloped fetus. Activated partial thromboplastin time (APTT) result was 32,2 seconds and Lupus anticoagulant (LA) qualitative test 36,3 seconds. APTT – LA/APPT was 1,13 (normal 0-1,2). These findings suggested that patient does not have antiphospholipid antibody syndrome or lupus anticoagulant syndrome.

The test for measuring the levels of folate, needed for the cell division as seen in the developing fetus, and vitamin B12, as it is important for nervous system, was ordered. Results of folates test was >45,3 nmol/l (normal 7-39 nmol/l). It showed slightly increased folate concentration in patient's blood. It might be the result of undergoing consumption of folic acid (400 micrograms per day). Karyotype of the patient was normal (46 XX) and the partners also (46XY).

The results of molecular genetic testing (Centre of Medical Genetics) showed a homozygous variation c.655C>T (known as C677T, p.A222V) in MTHFR gene located on chromosome 1 (egzone 4), causing increased levels of homocysteine in blood or, in other words, hyperhomocysteinemia.

Using NCBI variation viewer we found that the variation of MTHFR in the genome of our patient is one of 138 single nucleotide variants in MTHFR gene. Location of the mutation in the chromosome 1 is 1:11796321. It is a missense mutation that causes Alanine to change to Valine (p. Ala222Val). Therefore, it may be the cause of thermolabile type MTHFR deficiency. Clinical significance of this variation is benign. We consider that the variation of MTHFR gene in our case study might have been the cause of recurrent miscarriages.

#### *Genetic methodology description*

Peripheral blood was collected in acid citrate dextrose (ACD) tubes to prevent coagulation of blood samples. Genomic DNA was extracted from whole blood using the standard phenol-chloroform method. Detection of c.655C>T MTHFR polymorphism was performed by polymerase chain reaction (PCR) followed by HinfI restriction enzyme digestion. Briefly, we used the forward primer 5'-CCT TGA ACA GGT GGA GGC CAG-3' and the reverse primer 5'-GCG GTG AGA GTG GGG TGG AG-3' to amplify a 294 base pair (bp) fragment of the MTHFR gene. Each 25  $\mu\text{l}$  PCR reaction contained 2.5  $\mu\text{l}$  of 10X reaction buffer, 1.5 mmol MgCl<sub>2</sub> (Fermentas, Glen Burnie, MD), 2  $\mu\text{l}$  from 10 pmol of each primer, 0.2 mmol of the deoxynucleoside triphosphates, 1 U of Taq DNA polymerase (Fermentas), and 100 ng of genomic DNA template. The mixture was denatured at 95 °C for 10 min, and the PCR reaction was performed for 35 cycles in a thermocycler (Eppendorf AG, Hamburg, Germany) under the following conditions:

denaturation at 95 °C for 1 min, annealing at 65 °C for 30 s, and extension at 72 °C for 1 min. The final extension cycle of 72 °C was for 7 min. The PCR products were electrophoresed on an agarose gel (2%) to confirm the correct amplicon size. Restriction enzyme digestion was performed on PCR products using the *HinfI* restriction enzyme (Fermentas) following the suppliers protocol. After electrophoresis on agarose gel (3%) genotypes were detected according to the observed patterns.

#### DISCUSSION

Even though people with slight impairments in the folate metabolism usually do not have any complaints about their own health, it is observed that folates play an important role in pregnancy.

The most common form of hyperhomocysteinemia is caused by production of a thermolabile variant of MTHFR (methylene tetrahydrofolate reductase) with reduced enzymatic activity (WU *et al* 2012). This variant is a cytosine to thymine substitution which can be found at nucleotide 677 (C677T) of chromosome 1. The connection between the MTHFR C677T polymorphism and recurrent pregnancy loss in some populations was revealed by conducting a metaanalysis of the case-control studies. It is important to mention that the study of MTIRAOUI *et al* (2006), not only confirms the researchers conclusion that increased risk of RPL (recurrent pregnancy loss) is associated with MTHFR C677T genotype but also adds that the homozygosity of this variant is a risk factor for RLP regardless of the total homocysteine levels (HAGUE *et al* 2003). In some cases like our patient's, the level of homocysteine is only slightly elevated. Therefore, a different mechanism for fetal loss, less dependant on hyperhomocysteinemia, may exist. As a consequence, small changes in homocysteine level usually do not draw attention of a physician who is not well informed about the significance of MTHFR gene sequencing. It should be pointed out that recurrent pregnancy loss as well as other obstetric pathologies has been associated with a disturbed maternal and fetal homocysteine metabolism (ZETTERBERG *et al*, 2002). These findings suggest that testing of homocysteine levels is important to a great extent. Frequency of MTHFR C677T polymorphism is many times higher than rare mutations of monogenous diseases (such as Stargardt disease) (SERAPINAS *et al.*, 2013). The distribution of the C677T allele showed regional and ethnic variations. For example, the prevalence of the homozygous genotype was 10–12% in several areas in Europe (for example, Spain, France, and Hungary). However, the prevalence appeared to be lower (4% and 6%, respectively) in Finland, Helsinki and the northern Netherlands, whereas in some areas in southern Europe it was much higher (26% and 20% in Campania and Sicily, respectively) (WILCKEN *et al.*, 2003). Individuals homozygous for the C677T mutation had a 2.3  $\mu\text{mol/l}$  higher median homocysteine level compared to individuals with the wild-type allele (GEISEL *et al.*, 2001). So we suggest that stabilization of homocysteine levels in blood by treating homocysteinemia might be a possibility to prevent recurrent miscarriages in patients with homozygous variations in MTHFR gene. During embryogenesis folate requirement is high, hence the effect of one or more MTHFR mutated alleles may be detrimental for the fetus due to disturbed homocysteine metabolism. The potential protective role of periconceptional folic acid supplementation is also emphasized (STAM *et al*, 2005). Treatment with folic acid serves to enhance biochemical pathways that remove excess circulating homocysteine. The treatment is shown to have an effect of lowered plasma homocysteine concentration and increased remethylation and transmethylation in healthy subjects. The presence of ascorbic acid is required in folic acid conversion to its metabolically active form (tetrahydrofolic acid). It might be considered to prescribe vitamin C as a part of a treatment. Also,

bioavailability of naturally occurring folate in food is about 50%. Meanwhile, bioavailability of synthetic folic acid consumed with a meal ranges from 85 to 100% (MARON & LOSCALZO, 2009). To seek the best results of treatment, folic acid should be administered with meal. With a reference to the study of MARON & LOSCALZO (2009) we can claim that hyperhomocysteinemia can be treated by using combined folic acid and B-vitamin therapy which reduces homocysteine levels in blood. Overall, for the most effective treatment of homocysteinuria is a combination of folic acid, vitamin C and B group vitamin consumed with food. As a result of the treatment, decreased levels of homocysteine should be observed. In our case homocysteine concentration after treatment with B12 and B6 vitamins after two month decreased till 6.4  $\mu\text{mol/l}$ .

On the one hand certain MTHFR variations lead to the enzyme with reduced activity that influence elevated levels of homocysteine. On the other hand homocysteine level may be effected by various conditions (pregnancy, inflammation etc.). During inflammation soluble monocyte molecules CD14 may play role in metabolites upregulation through NF $\kappa$ B pathway (NITA *et al.*, 2007). So elevated levels of homocysteine may lead to higher risks for developing disease or having a recurrent miscarriage so MTHFR genotyping may provide information about potential causes of elevated homocysteine levels and ways to cope with it. Detection of MTHFR variations is essential in finding the causes of recurrent miscarriages, evaluating possible risks for a recurrent miscarriage, prescribing higher doses of folic acid and B group vitamins when needed. From the economic point of view, the test is cost-effective and would not become an economic burden for the health care system.

#### CONCLUSIONS

In the clinical case study, we have discussed, molecular genetic testing revealed a homozygous c.655C>T variation in the MTHFR gene of the patient with recurrent miscarriages. Even though the particular variation is thought to be benign, it might be the risk factor of hyperhomocysteinemia or elevated levels of homocysteine in blood. We recommend prescribing folic acid, methionine, betain and B group vitamins for treatment of hyperhomocysteinemia. Also, folic acid might be absorbed more effectively if consumed with a meal and using vitamin C supplements. Concluding from our case study, considering the prevention of a recurrent miscarriage, it is important to sequence MTHFR gene for variations even if blood samples show normal or borderline levels of homocysteine.

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**ZNAČAJ DETEKCIJE MUTANTA GENA MTHFR KOD PACIJENATA  
SA PONAVLJUĆIM POBAČAJIMA**

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Izvod

Homocisteine (methylenetetrahydrofolate reductase) je enzim pod kontrolom MTHFR gena lociranog na hromozomu 1. Mutacija u MTHFR genu može rezultirati u promeni metabolizma homocisteina i povećati rizik pobačaja. U nekim slučajevima poremećaj trudnoće može da se spreči davanjem folne kiseline i B grupe vitamina. Sekvenciranje MTHFR gena da se utvrdi mutacija obično je previđena od strane lekara i genetičkog savetovališta. Da bi se rasvetlio problem vršena su ispitivanja slučaja rekurentnog pobačaja kod pacijenta sa homozigotnom c. 655C>T promenom MTHFR gena.

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