GENETIC AND BIOCHEMICAL THROMBOSIS RISK MARKERS IN PREGNANCY. II. HOMOCYSTEINE METABOLISM

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Abstract

Hyperhomocysteinemia is considered a biomarker in thrombosis risk and adverse pregnancy outcomes. It results from alterations in the key methyl group metabolism of the human cells named “one-carbon metabolism”. This involves metabolites homocysteine, S-adenosylmethionine and S-adenosylhomocystein, whose functionality is linked with environmental factors (dietary cofactors intakes of the methylation reactions, such as B vitamins, including folate, methyl donors such as choline, methionine, betaine and alco microelements, including cations) and also with the endogenous, genetic factors (single nucleotide polymorphisms influencing the functions of genes controlling methylation and transsulfuration pathways such as methylentetrahydrofolate reductase - MTHFR, methionine synthase- MTR, methionine synthase reductase-MTRR, as well as DNA methyltransferase 3b- DNMT3b). Together, these genetic and epigenetic biomarkers are studied on a cohort of pregnant women with and without declared history of previous pregnancy problems in order to be integrated in a thrombophilia evaluation panel, suggesting certain prophylactic or treatment solutions additional to those already established from the coagulation biomarkers.

Keywords: thrombosis, one carbon metabolism, genetic polymorphism, DNA methylation, methyl donor, S-adenosylmethionine;

1. Introduction

Venous thrombosis, including deep vein thrombosis and pulmonary embolism, occurs worldwide at an annual incidence of about 1 per 1000 adults. Major risk factors for thrombosis, other than age, include exogenous factors such as surgery, hospitalization, immobility, trauma, pregnancy and the puerperium and hormone use, and endogenous factors such as cancer, obesity, and inherited and acquired disorders of hypercoagulation. As for hereditary factors, there are already reported differences in incidence of diagnosed venous thrombosis among ethnic groups: with lower rates in certain populations of the United States, Asian, Pacific Islands, and higher rates in African-Americans and Caucasians (R.H.WHITE [1], M. CUSHMAN [2]).
Aside from the hereditary factors, certain developmental and life-style factors are reported in the literature that determine increase of a metabolic imbalance predisposing to thrombosis through increases in coagulation potential (F.R. ROSENDAAL & al. [3]). Our previous report described an associative study of genetic polymorphism in genes controlling coagulation pathways and pregnancy outcomes and presents certain suggestions regarding the clinical significance and therapeutic attitude towards thrombophilia risk assessments (G.A. FILIPESCU & al. [4]). Recent research results have shown that additional knowledge of another major process in the cell controlling methylation and transsulfuration of vital cell molecules (lipids, proteins, glucides and nucelic acids) named „one - carbon metabolism” (OCM) is needed in order to establish a more complex diagnosis of thrombosis risk biomarkers and to point toward important endogenous molecules and specific environmental, nutritional factors for the management, prophylaxis and treatment schemes of those pregnancies affected by thrombophilia.

Therefore, aside from the coagulation, fibrinolysis and anticoagulation processes in blood, homocysteine, the central molecule of OCM, is added actually to coagulation metabolism key factors when the thrombosis risk is evaluated linked with the pregnancy adverse events. Homocysteine is generated from the metabolism of the amino acid methionine involving the external methyl groups supply from diet (Fig. 1). The OCM cycle is a network of biochemical reactions that provide specific metabolites required for critical intracellular processes such as methylation and trans-sulfuration. Impairments of these pathways are critical for cell homeostasis and hence have increasingly been observed in numerous disease states including cancer, neurodegenerative disease and adverse reproductive outcome (J. SEIHUB & J.W. MILLER [5], J.T. BROSNAN & al.[6]). Such impairments may result from environment influences (insufficient intakes of dietary cofactors such as folate and other B vitamins) as well as from endogeneous developmental and genetic causes (common polymorphisms in genes involved in OCM cycle (D.S. WALD & al. [7]).

Figure1 depicts a simplified schematic of the OCM cycle; it focuses on the role of key enzymes in the pathways, and their associated genes which are identified in brackets. The supply of methyl groups in this cycle of pathways is through the metabolism of dietary methionine. The OCM cycle begins with the activation of methionine by adenosine triphosphate (ATP) to produce SAM, the universal methyl donor. Trans- methylation of SAM occurs when a methyl group of SAM is transferred to an acceptor-like DNA, a process catalyzed by DNA methyl- transferases (DNMTs). The end product of this process, SAH, under normal physiologic conditions, is hydrolyzed to HCys and adenosine; HCys is further metabolized by two biochemical pathways: transsulfuration and remethylation. In the transsulfuration pathway, HCys is catabolized to form the amino acids cysteine and taurine in a series of irreversible reactions one of which is catalyzed by cystathione b-synthase (CBS). Remethylation implies transformation of Hcys in SAM using activated methyl group and enzyme activity (MTHFR, MTR, MTRR). Finally, SAM is involved in a methylation reaction again to process through the DNMT catalysis the DNA methylation in the cel, Maintenance of the genome stability is vital and is assured by these processes, hence any alteration in its pathways is critical for the cell homeostasis (D. JACOBSEN [8] S.J. James & al.[9]).

Knowledge about the regulation of the OCM pathways provided clues about the role of environmental and genetic factors upon the homocysteine level in blood; these are represented respectively by the diet, as the main supplier of methyl groups in cells for the methylation and transsulfuration reactions and genetic polymorphisms controlling the functionality of key genes encoding enzymes processing the methyl harboring compounds from diet (folates, betaine, choline).
Therefore, metabolic biomarkers of OCM pathways including homocysteine (HCys), S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) have often been used to represent the functionality of the cycle. Also, the genetic biomarkers may include the methylenetetrahydrofolate reductase (MTHFR) gene, whose product catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the methyl donor for the remethylation of homocysteine (Hcys) to methionine, methionine synthase-MS (MTR) and the methionine synthase reductase (MTRR) genes, encoding enzymes that maintain the methionine synthase enzyme in an active state for the remethylation of Hcys to methionine (Fig. 1) (D. LECLERC & al.[10], H. ZHU & al. [11], E. SLIWERSKA & A. SZPECHT-POTOCKA [12]).

Figure 1. One-carbon (homocysteine) and folate metabolisms are in close connection and depend on diet (folates) and the activity of the enzymes MTHFR, MTR, MTRR in the presence of vitamins B.

Consequently, genetic and nutritional factors play the major role in determining the functionality of the OCM cycle (V. HO & al. [13]). Genes encoding enzymes MTHFR and MTR may be added to the panel of biomarkers defining the OCM cycle that are metabolites of HCys, SAM and SAH. MTHFR C677T is an already established genetic determinant of HCys but less is known of its effect on SAM and SAH. The role of MTHFR polymorphism on HCys (V. DEKOU & al. [14]), SAM and SAH (L. DE VOS & al. [15]) was extensively investigated in certain population subsets, presenting pre-eclampsia in pregnancy, birth defects or miscarriages and also in cancer, developmental diseases like obesity and neurodegenerative conditions (L. DIWAKAR & al. [16], M. EL-SAMMAK & al. [17]). Conversely, the relationship between MTRR and HCys remains inconclusive, and its effect on SAM and SAH has only been previously investigated in a female-specific population, however inconclusively. Folate and vitamin B12 are essential substrate and cofactor of OCM; thus, consideration of gene–nutrient interactions may clarify the role of genetic determinants of HCys, SAM and SAH in the thrombophilia risk assessment panel (J.D. FINKELSTEIN [18], A. FREDRICSEN & al. [19], D. J. GAUGHAN & al. [20], J. GEISEL & al. [21]).

This research has been started in order to examine the effects of MTRR, MTR, DNMT3b and MTHFR polymorphisms on plasma HCys, SAM and SAH concentrations. These results were compared with those obtained for the same group of pregnant women tested for the coagulation
factors in order to estimate the thrombosis risk (A.G. FILIPESCU & al. [4]). Also, gene–gene and gene–nutrient interactions were considered in order to stratify the thrombosis risk and to establish a personalized prophylaxis or treatment scheme during pregnancy.

2. Materials and Methods

This study included 150 healthy women presented for the management of their pregnancy at Elias Emergency Hospital Room, during 2012-2014. 80 of them presented with a history of birth defects, pregnancy adverse events such as abortion, stillbirths, miscarriages and preeclampsia and they were evaluated for the level of thrombosis risk by genetic and biochemical biomarkers in order to establish a proper prophylactic or therapeutic approach. The rest of patients declared a history of no adverse pregnancy outcomes and has been considered as control, but still having a potential for thrombosis risk based on their laboratory markers and hence suited for the prophylactic scheme. Each patient has been enrolled in this study based on their signed informed consent. The tests were approved by the bioethical Committee of the Hospital.

Peripheral blood samples were collected from all pregnant women. Blood samples (5 mL) were collected in EDTA tubes and genomic DNA was isolated from lymphocytes in whole blood using the Qiagen minispin blood kit, according to the manufacturer protocol, and was stored at -20°C until genotype analysis was performed. Also, for the homocysteine metabolism assays, blood was collected on heparine (1mL) and processed for the extraction and analysis of Hcys, SAM and SAH according to biochemical protocols.

Genotyping technique implied DNA amplification and reverse hybridization on strips. Qiagen mini-spin blood kit was used for rapid DNA extraction; an Euroclone RhymaTest OMO primer kit for PCR amplification in multiplex system was used, followed by using the same company strip system for the realization of the RFLP reverse blotting of the amplicons, their fixation and color revealing after labeling with avidin probes provided by the same kit. A diagram (Fig. 2) on a strip with bands corresponding to each allele form was used for comparison of the coloured strips and recording the allele variation results (Table 1).

Biochemical assays. Homocysteine was assayed by HPLC method at Bioclinica Medical Center. S-adenosylmethionine and S-adenosylhomocysteine were assayed by HPLC at National Institute for Biological Sciences Bucharest, Department of Bioanalysis, according to a protocol described elsewhere (C. ALBU & al. [22], C. BIRSAN & al. [23]).

3. Results and Discussions

In this study, we investigated genetic determinants (MTRR, MTR and DNMT3b polymorphisms) of Hcys, SAM and SAH concentrations in a group of 150 healthy pregnant women, of which, 80 declared a history of former adverse pregnancy events linked with thrombosis in order to complete the previously published (A.G. FILIPESCU & al. [4]) picture of coagulation processes (factor II, FactorVLeiden as genetic factors and protein S and protein C as biochemical factors).

The results of the genetic approach are shown in Figure 2. The inverse blotting diagrams on strips of the polymorphic variation in the studied genes were rapidly and easily processed as compared with the same processing step used in other PCR-RFLP methods common for genotyping single nucleotide polymorphisms (H. B. RASMUSEN [24]).

An example of genotype variance in a group of eight pregnant women suspected for thrombophilia shows (Table 1) great variance among the genotypes of MTHFR C677T, MTHFR A1298C, MTRRA66G, but negligible variance in DNMT3B-149C>T genotypes and
no variation in MTR A2756G genotypes. This variance was proved also at the level of all patients included in the study (of the total of 80 patients suspected for thrombophilia and having previously declared pregnancy problems, 69% presented MTHFR C677T mutation, 39% MTHFR A1298C mutation, 56% MTRRA66G mutation, 14% DNMT3B-149C>T mutation and only cca 6% MTR A2756G mutation). Similar frequencies were however obtained at the entire cohort level, of 150 pregnant women, the group of 70 individuals having no declared previous pregnancy problems.

Table 1. Example of genotype variance in a group of 8 pregnant women for genes controlling folate and one-carbon metabolism, that were suspected for thrombophilia.

<table>
<thead>
<tr>
<th>Patient</th>
<th>MTHFR C677T</th>
<th>MTHFR A1298C</th>
<th>MTR A2756G</th>
<th>MTRR A66G</th>
<th>DNMT3B -149 C&gt;T</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>WW</td>
<td>MM</td>
<td>WW</td>
<td>WM</td>
<td>WW</td>
</tr>
<tr>
<td>2</td>
<td>WM</td>
<td>WM</td>
<td>WM</td>
<td>WM</td>
<td>MM</td>
</tr>
<tr>
<td>3</td>
<td>WM</td>
<td>WW</td>
<td>WW</td>
<td>WM</td>
<td>WW</td>
</tr>
<tr>
<td>4</td>
<td>MM</td>
<td>WW</td>
<td>WW</td>
<td>WM</td>
<td>MM</td>
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<tr>
<td>5</td>
<td>WM</td>
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<td>6</td>
<td>WW</td>
<td>WM</td>
<td>WW</td>
<td>WW</td>
<td>WW</td>
</tr>
<tr>
<td>7</td>
<td>WW</td>
<td>MM</td>
<td>WW</td>
<td>WM</td>
<td>WM</td>
</tr>
<tr>
<td>8</td>
<td>WW</td>
<td>WM</td>
<td>WW</td>
<td>WM</td>
<td>WW</td>
</tr>
</tbody>
</table>

The variable concentrations in blood of Homocysteine (Hcys) and additional metabolites, SAM, SAH released in the OCM pathways are represented in Table 2 with the frequency distribution and percentages for select characteristics of the study population.

The results showed that in spite an almost unchanged value for Hcys, there are corresponding variable SAH and SAM values. Therefore, a more sensible evaluation of the thrombosis risk may be suggested through the use of additional variation in the Hcys precursors involved in methylation reactions. A decrease in SAM value concomitant with the increase in SAH value suggested a decrease in methylation potential, that mathematically showed a decreased of SAM to SAH ratio. This may be explained by an increase in the tendency for the further, yet non-measurable, Hcys level, that may result in an increase in the thrombosis risk indicator. The nutritional evaluation of the individuals was not rigorously approached: however, it proved the implication of dietary factors (vitamin B supplementations and micronutrient intake) in the case of high SAM to SAH ratios and high SAM values maintenance concomitant with low Hcys values). Therefore, it may be suggested that the thrombosis risk may vary depending on such environmental, nutritional factors that may be included in a prophylactic scheme in the case of suspected thrombophilia at pregnant women.

In this study, we investigated genetic determinants (MTRR, MS and DNMT3b and MTHFR polymorphisms) of Hcys, SAM and SAH concentrations in a cohort of pregnant women, with the previously declared history of pregnancy problems subgroup and the control “healthy” subgroup. Beyond genetic control, disturbances in nutrient intakes and metabolism may be further studied that may interfere with homeostasis of the OCM cycle and, in turn, Hcys, SAM and SAH levels. Thus, accordingly, interactions between polymorphisms and relevant nutrient cofactors involved in OCM (serum folate and vitamin B12) on Hcys, SAM and SAH concentrations are suggested for further examinations. Investigating the influences of genetic and nutritional factors on metabolites of OCM will contribute to a better understanding of the role of OCM in the alteration of blood coagulation processes and
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thrombosis risk in pregnancy. A general conclusion may be withdrawn that, in order to evaluate properly the individual thrombosis risk, none of the genetic and biochemical markers have to be considered alone, but in combination and including also the environmental (nutritional) aspects.

Figure 2a,b,c. 2a.Comparative diagram for the identification of allele oligonucleotide after reverse hybridization and colouring steps. 2b,c. The diagram of the oligonucleotide DNA bands colored by amplicones after reverse hybridization on strips which is compared with the standard diagram representing the genotypes for each gene. A. Normal pregnancies. B. Pregnancies with certain adverse events. The red marker-control; The genes of the one carbon metabolism controlling the homocysteine level are MTHFR, MTR, MTRR, DNMT3b.

Table 2. Variation in HCys/SAM/SAH levels in the pregnant women groups (suspected and nonsuspected for thrombophilia).

<table>
<thead>
<tr>
<th>Metabolic Parameter</th>
<th>Subgroup of declared history of pregnancy problems</th>
<th>Subgroup of no declared history of pregnancy problems</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Frequency %/intervals of variation</td>
<td>Frequency %/intervals of variation</td>
</tr>
<tr>
<td>SAM level (µMol/L)</td>
<td>23% 0.1-1.5 63% 1.5-3.9 14% 4.0-7.6</td>
<td>73% 1.5-3.9 27% 4.0-7.6</td>
</tr>
<tr>
<td>SAH level (µMol/L)</td>
<td>43% 0.01-1.0 38% 1.0-2.0 19% 2.0-6.7</td>
<td>65% 0.01-1.0 35% 1.0-2.0</td>
</tr>
<tr>
<td>SAM to SAH ratio</td>
<td>10% 0.11-1.5 9% 1.8-3.81 81% 4.5-22</td>
<td>36% 1.8-3.81 63% 4.5-22</td>
</tr>
<tr>
<td>HCys level (µMol/L)</td>
<td>5-8</td>
<td>5-6</td>
</tr>
<tr>
<td>Total subgroup</td>
<td>80</td>
<td>70</td>
</tr>
<tr>
<td>Total cohort</td>
<td>150</td>
<td></td>
</tr>
</tbody>
</table>

According to literature, the role of MTHFR together with MTR, MTRR and DNMT3b polymorphisms on HCys, SAM and SAH (J.D. FINKELSTEIN [18], A. FREDRIKSEN & al. [19], D.J. GAUGHAN & al. [20], J. GEISEL & al. [21]) was extensively investigated indicating a general association with numerous pathological conditions from pregnancy
adverse events, to cancer, developmental illness etc. The common polymorphism in the MTHFR gene (MTHFR C677T) has been discussed in the previous article (A.G. FILIPESCU & al. [4]) its functional is referring to the decrease in enzymatic activity by 20–30 % (W. ILAN & al. [25]). For the MTRR gene, an A2756G polymorphism has been described, that is defined by a change of aspartic acid to glycine at position 2756, whose functional significance is currently unknown (D. L. HARMON& al. [26]).

Several studies have demonstrated that elevation of homocysteine, is a risk factor for cardiovascular diseases, stroke and venous thromboembolism. Substantial evidence links elevated homocysteine levels to thrombosis via several mechanisms such as increased tissue factor expression, attenuated anticoagulant processes, enhanced platelet reactivity, increased thrombin generation, augmented factor V activity, impaired fibrinolytic potential, and vascular injury, including endothelial dysfunction. The molecular mechanisms underlying pro-thrombotic actions of homocysteine are incompletely deciphered, however certain key processes are evidenced: oxidative stress, DNA hypomethylation, and proinflammatory effects. Hyperhomocysteinemia is currently considered a relatively weak prothrombotic factor and moreover, it can be modified by administration of vitamins: these ones reduce homocysteine levels acting as cofactors of the enzymes involved in the methionine metabolism, and hence may decrease the risk of arterial and/or venous thromboembolic events (B. MARON, J. LOSCALZO [27], R.T. EBERHARDT & al. [28], K.S. MC CULLY [29], H. REFSUM & al. [30], I. M. GRAHAM & al. [31]).

The link between the HCys level and thrombosis was recently suggested in the recent literature reports. Over the past 30 years, evidence from many in vitro and in vivo studies supports an association between mild to moderate hyperhomocysteinemia and vascular dysfunction and disease (J. PERLA-KAJAN & al. [32]). A key mechanism that predisposes to vascular disease in hyperhomocysteinemia is endothelial dysfunction. Hyperhomocysteinemia produces a prothrombotic state (A. T. JAKOVINA & al. [33]), some mechanisms for which include enhanced platelet activation, enhanced coagulation (likely as a consequence of increased tissue factor expression, and attenuated fibrinolysis. Two recognized mechanisms for reduced fibrinolysis include posttranslational modification of fibrinogen by homocysteinylolation, rendering the fibrin derived from fibrinogen relatively resistant to plasmin, and increased activity of thrombin-activatable fibrinolysis inhibitor (J. PERLA-KAJAN & al. [32]). A number of studies have shown a pregnancy related decrease in plasma homocysteine concentration in healthy women (M. DEN HEIJER & al. [34], L. J. LANGMAN & al. [35], A. UNDAS& al. [36]).

An association between elevated levels of homocysteine and pregnancy related disorders such as pre-eclampsia, early pregnancy loss, abruptio placentae have been reported (S.C. KALHAN & al. [37]). A recent systematic review and meta-analysis by Hogeveen and colleagues concluded that higher maternal total homocysteine concentrations are associated with a small increased risk of small for gestational age offspring. It corresponded to increased in maternal total homocysteine (M. HOGEVEEN & al. [38]).

Our results and those of others support the involvement not only of MTHFR enzyme, but also MTRR, MS and DNMT3b polymorphisms in the complex risk picture for thrombophilia.

4. Conclusions

We examined the effects of MTRR, MTR, DNMT3b and MTHFR polymorphisms on plasma HCY, SAM and SAH concentrations. Main effects of MTR and MTHFR polymorphisms on HCys concentrations were observed; however, no gene–gene or gene–
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nutrient interactions were found and therefore we suggest further study the involvement of other biochemical markers in blood, such as vitamin B level, cations and inflammation factors, such as interleukins and reactive protein C. Also, a transcriptomic approach of interleukin genes may be more indicative of inflammatory processes linked with alteration of processes in vascular coagulation. No association was observed for SAM. For SAH, interactions between MTR and MTHFR polymorphisms, and MTHFR polymorphism were found. The findings of this research provide evidence that HCys and SAH, biomarkers of OCM, are influenced by genetic and nutritional factors and their interactions. It is generally accepted that mild hyperhomocysteinemia is quite common in population and variation in this biomarker has to be considered not only as linked with genetic causes (gene polymorphisms in the previously described genes controlling the one carbon metabolism: genes encoding enzymes MTHFR, MTRR, MS and DNMT3b), but also in association with nutritional deficiency, certain medications. The role of genetic factors in determining HCys levels is well established in contrast to investigations of genetic determinants of SAM and SAH. However, in our work the Hcys level remained almost constant and SAM/SAH levels varied according probably to genetic causes and nutritional states. In conclusion, beyond genetic control, disturbances in nutrient intakes and metabolism can interfere with homeostasis of the OCM cycle and, in turn, HCys, SAM and SAH may be monitored. Investigating the influences of genetic and nutritional factors on metabolites of OCM will contribute to a better understanding of the role of OCM in the pathogenesis of thromboembolism and other adverse pregnancy outcomes.

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