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RESEARCH ARTICLE

MTHFR - Ala222Val Effects on Metabolic Syndrome Progression

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Objective: Methylene-tetrahydrofolate reductase (MTHFR) is involved in adapting metabolism to environmental challenges by various mechanisms, including the control of gene expression by epigenetic and post-translational changes of transcription factors. Though a metabolic syndrome candidate gene, association studies of its common polymorphism rs1801133 (MTHFR-Ala222Val) remain inconclusive with important ethnic differences, and the effect on disease progression was not addressed. **Methods**: 307 middle-aged metabolic syndrome patients in a central Romanian hospital setting were investigated metabolically, and genotyped by PCR-RFLP. Disease progression was assessed by the age of onset of metabolic components, as well as development of non-alcoholic fatty liver disease and atherosclerotic complications. **Results**: The minor allele frequency of rs1801133 was 30.13%. Metabolic parameters showed no statistically significant differences according to genotype, but variant carriers developed dysglycemia and dyslipidemia earlier (53.28±10.8 vs 59.44±9.31 years, $p<0.05$ and 58.57±11.31 vs 64.72±10.6 years, $p<0.1$).While the polymorphism did not influence hepatic complications, an inverse association was found for manifest atherosclerosis (OR=0.49, p=0.006, 95%CI:0.29-0.81), which may be folate-status dependent, and needs further investigations. Simultaneous analysis with transcription factor polymorphisms (rs1801282, rs8192678) showed that the more protective genotypes were present the later metabolic disturbances developed, and in the presence of the other two variants the apparent protective cardiovascular effect disappeared. **Conclusions**: The common functional polymorphism rs1801133 may influence metabolic syndrome progression, the age of onset of components and development of atherosclerotic complications. Besides simple additive effects, complex mitigating and aggravating variant interactions may exist, and the protective or predisposing outcome may depend on modifiable environmental factors.

Keywords: metabolic syndrome X, atherosclerosis, non-alcoholic fatty liver disease, methylenetetrahydrofolate reductase, genetic polymorphism

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Introduction

Since the human genome is considered to be relatively unchanged from Stone Age, it could be deduced that the diabesity epidemic may be related to an inadequate interaction between inherited genetic polymorphisms associated with a survival advantage in long starving periods of the past and significantly changed current sedentary lifestyle and food abundance, an explanation formulated by Neel's thrifty hypothesis. Obesity and insulin resistance are considered central disturbances in the development of the much debated clinical entity known as the metabolic syndrome (MetS). Molecules acting as transcription factors, their co-activators and epigenetic regulators as well as posttranslational modifiers, responsible for the direct or indirect control of gene expression at various levels on short and long term, involved in fatty acid oxidation, adipogenesis and lipogenesis play a crucial role in the adaptation of metabolism to environmental challenges, thus their genes are important candidates of disease development.

MTHFR (methylene-tetrahydrofolate reductase) is a key enzyme of folate metabolism, controlling the proportional use of one-carbon units in nucleic acid synthesis and methylation - a major mechanism of epigenetic control and posttranslational changes, as well as methionin synthesis, involving the detoxification of homocysteine associated with increased oxidative stress. The most common and potentially functional polymorphism rs1801133 (MTHFR – Ala222Val) was widely investigated in type 2 diabetes, but less studied, with even more conflicting results in MetS, or non-alcoholic fatty liver disease (NAFLD) and atherosclerotic cardiovascular disease (CVD) associated with it.[1, 2] While previous studies targeted the assessment of the associated disease risk, the effect on progression was not investigated based on a Pubmed search. Given the controversial findings and lacking data, as well as potential practical implications of a genotype-based case management, we proposed to explore the effect of the polymorphism on the development of MetS components and complications, to our best knowledge uninvestigated in the local population. We hypothesized, that MTHFR polymorphism could influence MetS development by various mechanisms including the epigenetic control at DNA and histone level or by post-translational changes at protein level of transcription factors central for adipogenesis, lipid and glucose metabolism. Preclinical data suggest that adequate co-activator function of PPARGC1A (peroxisome proliferator-activated receptor-γ coactivator 1-α), including its interaction with PPARG2 (peroxisome proliferator-activated receptor-γ), depends on MTHFR activity.[3, 4] Joint effect of the most common polymorphisms of these three master regulators of metabolism by direct and indirect control of gene expression, however, was not previously addressed.

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Methods

307 MetS patients in a central Romanian region, diagnosed by the International Diabetes Federation proposed criteria, were recruited from Mureş County Emergency Hospital, and included in the study after obtaining informed written consent, according to the protocol approved by the institutional Ethics Committee.

Disease progression was assessed by the age of onset of metabolic components and development of non-alcoholic fatty liver disease and atherosclerotic complications. NAFLD was assessed as an elevated alanin transaminase level (>30IU/L in males, >19IU/L in females), and/or increased fatty liver index (>60, calculated according to the formula developed by Bedogni et al, 2006: $e^{0.953*\log_e(\text{triglycerides}) + 0.139*\text{BMI} + 0.718*\log_e(\text{GGT}) + 0.053*\text{waist circumference} - 15.745)} / (1 + e^{0.953*\log_e(\text{triglycerides}) + 0.139*\text{BMI} + 0.718*\log_e(\text{GGT}) + 0.053*\text{waist circumference} - 15.745)} \times 100$), and/or a positive diagnosis; all cases with liver disorder of known other etiology (i.e. toxic or infectious) were excluded. CVD was considered in clinically manifest atherosclerosis diagnosed as peripheral arteriopathy and/or cerebro- and/or cardiovascular disease.

Genotyping was done by PCR-RFLP using the following primer pairs (Eurogentec) and restriction enzymes (FastDigest, Thermo Scientific):

- 5'-CATCCCTATTGGCAGCTTAC-3'/5'-GACGGTGCGGTGAGAGTG-3', Hinf1 (rs1801133-MTHFR/Ala222Val);
- 5'-CAAGTCCTCCAGTCCTCAC-3'/5'-GGGGTCTTTGAGAAAATAAGG-3', MspI (rs8192678-PPARGC1A/Gly482Ser);
- 5'-GCCAATTCAAGCCCAGTC-3'/5'-GATATGTTGCAGACAGTGTATCAGTGAAGGAATCGCTTTCCG-3', Bsh1236I (rs1801282-PPARG2/Pro12Ala).

Amplification took place after an initial denaturation at 95^{0}C–5 min, in 38 cycles consisting of 95^{0}C–40 sec, 57/58/60^{0}C–40 sec and 72^{0}C–40 sec, ended with a final extension 72^{0}C–5 min. Digestion was carried out at 37^{0}C in 5 min, followed by electrophoresis in 2% agarose gels stained with Ethidium Bromide.

Statistical analysis was done with IBM SPSS Statistics 20, considering results statistically significant if $p < 0.05$ (two-tailed). Data are expressed as mean±standard deviation (SD), and compared by one-way ANOVA. Categorical variables are expressed as percentage, and examined by Fisher's exact test.

Results

307 MetS patients (61.62±10.59 years old; 47.88% males, 52.11% females; 57.01% from urban and 42.99% from rural area) were investigated as summarized in Table I.

Minor allele frequencies of rs1801133, rs8192678 andrs1801282 in the MetS patients investigated were 30.13, 32.89 and 15.79%, respectively; all polymorphisms were in Hardy-Weinberg equilibrium (p>0.05). When calculating with genotype combinations, homo- and heterozygous variant carriers were interpreted together according to the dominant model (rs1801133: Ala/Val+Val/Val; rs8192678: Gly/Ser+Ser/Ser; rs1801282: Pro/Ala+Ala/Ala).

Metabolic parameters according to the possible predisposing genotype combinations are presented in Table II.

To assess genotype dependent MetS development and progression, the age of onset of metabolic disturbances, as well as the risk of NAFLD and CVD was analyzed (Table III and IV).

Discussion

Progress in genomics calls for a better characterization of genetic variation, confirmation of functional consequences, assessment of gene – gene and environment interactions, testing disease associations in various ethnicities. Possibilities to investigate joint effects of polymorphisms remain limited. Risk scores have little practical utility, especially in older people for adult onset multifactorial disorders be-

Table I. Clinical characterization

Clinical parameter	Mean±SD
Body Mass Index-BMI (kg/m^2)	
Males	29.9±4.77
Females	30.82±7.05
Waist Circumference (cm)	
Males	107.43±14.25
Females	98.11±14.03
Fasting Glucose (mg/dL)	125.44±43.68
Systolic Blood Pressure (mmHg)	148.12±21.54
Diastolic Blood Pressure (mmHg)	87.13±12.01
Triglyceride (mg/dL)	203.24±117.87
HDL-Cholesterol (mg/dL)	
Males	48.95±12.81
Females	50.55±14.95
Total Cholesterol (mg/dL)	210.7±54.44
Alanine transaminase (IU/L)	
Males	30.92±15.21
Females	29.58±12.61
Fatty Liver Index	83.67±17.8

Table II. MetS components according to the suspected predisposing genotype combinations*

	No predisposing variant	rs1801133	rs1801133 + rs8192678	rs1801133 + rs1801282	rs1801133 + rs1801282 + rs8192678
Body Mass Index (kg/m^2)	28.57±4.84	33.08±10.65	31.05±6.83	30.26±6.58	29.63±5.19
Waist Circumference (cm)	98.35±9.81	107.31±12.59	108.7±12.61	101.72±14.92	101.7±15.78
Fasting Glucose (mg/dL)	112.52±23.6	124.28±41.3	115.24±30.88	128.70±48.2	123.54±41.65
Systolic Blood Pressure (mmHg)	150.1±20.9	145.48±21.65	149.5±18.62	148.59±19.49	146.07±22.3
Diastolic Blood Pressure (mmHg)	81.8±7.76	85.79±12.67	93.49±12.9	87.83±12.18	90.32±11.37
Triglyceride (mg/dL)	188.18±77.9	172.54±129.65	226.6±93.5	190.6±138.4	267.98±110.15
HDL-Cholesterol (mg/dL)	41.14±6.61	52.47±18.22	47.29±7.48	47.06±20.19	45.8±15.77

*p>0.05

Table III. Onset of MetS components according to the genotype*

		Type 2 diabetes		High Blood Pressure		Dyslipidemia	
		Mean±SD	p	Mean±SD	p	Mean±SD	p
rs1801133	Ala/Val or Val/Val	53.28±10.8	0.007	54.95±13.57	0.32	58.57±11.31	0.06
	Ala/Ala	59.44±9.31		52.36±11.58		64.72±10.6	
rs1801133 + rs8192678	Ala/Val or Val/Val + Gly/Ser or Ser/Ser	55.13±12.02	0.51	54.88±11.27	0.79	58.38±13.13	0.07
	Ala/Ala + Gly/Gly	56.98±9.94		55.68±9.69		64.32±9.7	
rs1801133+ rs1801282	Ala/Val or Val/Val + Pro/Pro	52.38±10.25	0.19	53.34±11.7	0.69	58.48±10.95	0.28
	Ala/Ala (MTHFR) + Pro/Ala or Ala/Ala (PPARG2)	56.73±8.83		55.22±15.65		62.15±10.47	
rs1801133 + rs8192678 + rs1801282	Ala/Val or Val/Val + Gly/Ser or Ser/Ser + Pro/Pro	55.88±10.93	0.21	57.94±10.75	0.38	59.05±12.65	0.25
	Ala/Ala (MTHFR) + Gly/Gly + Pro/Ala or Ala/Ala (PPARG2)	61.60±7.76		61.17±6.24		64.14±8.72	

*Age of onset in years

Table IV. Risk of NAFLD and CVD according to the genotype

		NAFLD			CVD		
		OR	p	95%CI	OR	p	95%CI
rs1801133	Ala/Val+Val/Val vs Ala/Ala	0.54	0.3	0.18-1.6	0.49	0.006	0.29-0.81
rs1801133 + rs8192678	Ala/Val or Val/Val + Gly/Ser or Ser/Ser vs Ala/Ala + Gly/Gly	0.75	0.46	0.36-1.55	0.63	0.22	0.3-1.28
rs1801133 + rs1801282	Ala/Val or Val/Val + Pro/Pro vs Ala/Ala (MTHFR) + Pro/Ala or Ala/Ala (PPARG2)	1.29	0.53	0.58-2.84	0.57	0.25	0.25-1.29
rs1801133 + rs8192678 + rs1801282	Ala/Val or Val/Val + Gly/Ser or Ser/Ser + Pro/Pro vs Ala/Ala (MTHFR) + Gly/Gly + Pro/Ala or Ala/Ala (PPARG2)	1.5	0.45	0.57-3.92	0.64	0.46	0.23-1.79

cause of lifelong exposure to environmental risk factors. Study of genetic variants influencing disease progression could provide perhaps more valuable information, and by analyses in the same age group and region, long-lasting effects of confounding factors could be better accounted for.

Molecules acting as transcription factors, their co-activators and epigenetic regulators as well as posttranslational modifiers, involved in the direct or indirect control of gene expression, on short and long term, regulating genes involved in metabolism play a crucial role in its adaptation to environmental challenges. The functional polymorphism rs1801133 (C677T; Ala222Val) of MTHFR leads to a thermo-labile form with altered substrate/cofactor affinity and enzyme activity decrease to 1/3 and 2/3 in hetero- and homozygous carriers.[5] Folic acid supplements compensate variant dysfunction, though preventive utility proved in congenital anomalies is uncertain in atherosclerosis. rs1801133 associates with increased homocysteine levels, and was reported inconsistently in association with MetS components and other common disorders (e.g. Alzheimer disease, depression), mainly in Asians and less in Caucasians.[1, 2, 6] Moreover, second-generation antipsychotics related MetS development appears to depend on the polymorphism.[7] Ultraviolet exposure-related lower folate levels may be genotype specific, and could account for ethnic differences.[8] Protective effects reported in cancer, renal failure or NAFLD, may suggest heterozygosis advantage.[9]In the local population, we observed a relatively high variant frequency in both patients and healthy controls, with no significantly increased MetS risk associated.[10] Hyperhomocysteinemia is often considered part of the MetS. Hyperinsulinemia was reported to decrease MTHFR activity, and hyperhomocysteinemia to hinder insulin signaling.[11, 12] We speculated that polymorphism reduced enzyme activity and insulin resistance in MetS could be mutually aggravating, but accentuated insulin resistance assessed by HOMA and QUICKI indices, though increased in T allele carriers in both patients and control, did not differ significantly as compared to CC in our population.[13] As far as MetS disease progression is concerned in patients, variant carriers appear to develop dysglycemia and to a lesser extent dyslipidemia significantly earlier (53.28±10.8 vs 59.44±9.31 years, $p<0.05$ and 58.57±11.31 vs 64.72±10.6 years, $p<0.1$).

The effect on MetS-associated NAFLD and CVD may be indirect or direct, by metabolic changes, inflammation and oxidative stress. Clinical experience suggests that NAFLD is the hepatic manifestation of MetS, resulting from an imbalance between fat supply, formation and consumption, as well as pro- and anti-oxidant action; in fact, triglyceride accumulation in the beginning could be protective by decreasing free fatty acid accumulation and the associated oxidative stress.[14] According to the two-hit hypothesis, first steatosis develops, and then hepatitis and fibrosis. While steatosis develops in the majority of obesity cases, progression to hepatitis and fibrosis is rarer, and could have genetic causes. Among the candidate genes, MTHFR was also studied. Hyperhomocysteinemia considered a risk factor for liver disease by increased oxidative stress, activation of pro-inflammatory factors and altered intracellular lipid metabolism, could explain NAFLD association with MTHFR-Val, but was not equivocally demonstrated: it may not exist in Caucasians, and could characterize only subjects homozygous for the variant.[15] In the dominant model we applied, no such effect was seen, similar to neighboring populations [16]. The conflicting results could be related to folate status, as suggested in CVD. rs1801133 is viewed as a proxy for

hyperhomocysteinemia-a CVD risk factor associated with increased oxidative stress, but genetic association studies are few and inconclusive.[17, 18] Our unexpected results may confirm the inverse association between the homozygous genotype for the variant and CVD mortality found in Caucasian populational studies, despite higher homocysteine levels. Possible explanations cited include publication bias, methodological problems and folate status, since lower CVD-mortality may characterize persons with adequate folate intake. Accordingly, geographic and dietary differences might explain the contradictory findings and the significant risk reduction we found in variant carriers despite an earlier onset of metabolic disturbances considered CVD risk factors, but require further investigations, including folate status assessment. Confirmation would emphasizethe importance of genetics and gene-environment interactions in patient management, and may signify the potential inversion of variant effect from predisposing to protective by lifestyle interventions, diet and vitamin supplements. Moreover, the polymorphism was shown to require further tailoring of dietary interventions, i.e. adequate protein intake in case of a hypocaloric diet to prevent sarcopenia in T allele-carriers.[19]

Common functional variants of metabolic master regulators individually or jointly, by independent additive effect within a polygenic system or complex interactions and inter-dependent activity, may influence the MetS phenotype. Lipotoxicity is opposed via adipose tissue remodeling - increased storage capacity by adipocyte hypertrophy and hyperplasia, or via increased use by lipid oxidation. [20, 21] According to the adipocentric view, PPARG is an important MetS candidate. As a transcription factor, PPARG2 controls the activity of genes involved in adipocyte development and fat storage, lipid metabolism and inflammation. By expansion of the subcutaneous adipose tissue, however, lipids accumulate selectively in hormonally less sensitive depots, while there is no change in visceral adiposity with direct access to portal circulation.PPARGC1A described initially as a PPARG co-activator, is a multifunctional regulator of various transcription factors. It plays a central role in the regulation of metabolism and energy homeostasis, coordinating metabolic pathways in response to environmental requirements such as food supply, exercise or temperature. It is involved in the control of mitochondrial biogenesis, adaptive thermogenesis, adipocyte differentiation, fatty acid β-oxidation and glucose metabolism.[21, 22] PPARGC1A and PPARG2 functions are closely related: PPRAGC1A regulates PPARG2, and major metabolic effects of the co-activator are carried out by the nuclear receptor. Relationships of MTHFR with PPARGC1A, PPARG2 and MetS appear manifold, and are little studied. Adequate PPARGC1A activity depends on post-translational changes. A possible mechanism involved in Barker's early origins of adult onset disorders hypothesis could be the imbalanced methylation-acetylation of PPARGC1A dependent on MTHFR.[4] Later in life rs1801133-associated hyperhomocysteinemia causing enhanced PPARGC1A nitrotyrosylation could further impair co-activator function and interaction with PPARG2 [3]. Additionally, PPARG ligation reduces homocysteine levels, and in PPARG2 variant carriers homocysteine levels were found higher,[23] suggesting a bi-directional interaction. Common variants of these metabolic master genes have been rarely studied together, and based on a Pubmed search the combined effect of the three polymorphisms has not been addressed yet, despite interactions suggested by preclinical research and potential practical utility based on limited clinical data in assessing disease susceptibility, prediction of progression or response to interventions. [24] rs1801282 is a missense mutation (Pro12Ala) in the ligand-independent activation domain of PPARG2, associated with reduced DNA-binding and 30% decrease in trans-activation.[25] rs8192678 is a missense mutation (Gly482Ser) of PPARGC1A, and though apparently it doesn't alter binding sites for known transcription factors, reduced activity could be explained by impaired interaction efficacy.[26, 27] The mutation-containing exon encodes for a domain that appears to interact with PPARG, so a conformational change might reduce binding affinity. Protein structures resulting from the amino-acid sequence changes don't offer clear answers about interactions. In PPARG2, the polymorphism is contained within the variable A/B region on the N-terminal end responsible for ligand-independent trans-activation, while the F region on the C-terminal end appears important for interaction with co-activators. Functional consequences of the polymorphisms on interaction are contradictory. While some found that neither substitution influenced target gene transcription in vitro,[26] others demonstrated that PPARG2 activation by PPARGC1A is compromised in the presence of Ala,[28] confirmed by epidemiologic observations in breast cancer related to alcohol consumption [29]. Petersen et al. suggested an interaction characterized by a Pro allele-specific effect of the PPARG-PPARGC1A complex, but Ruchat et al. reported an independent effect in non-diabetic subjects undergoing glucose tolerance test, higher fasting insulin and HOMA-IR in carriers of both Gly and Ala compared to Ala carrier-Ser/Ser persons, with no difference in Pro/Pro subjects [30].The suggested PPARGC1A co-activator function and interaction with the nuclear receptor PPARG2 dependent on normal MTHFR activity could be impaired in the presence of rs1801133, and presumably the effect could be further modulated in carriers of rs8192678 and rs1801282. Though simultaneous presence of polymorphisms did not further aggravate onset of metabolic disturbances, the age of onset of dyslipidemia in case of bothrs1801133 and rs8192678 remained reduced to the same extent. In the presence of rs8192678 and rs1801282, however, the apparent significant protective effect on atherosclerotic complication development of the MTHFR variant disappeared, possibly mitigated by the pro-atherogenic effects of the transcription factor vari-

ants through metabolic changes, mitochondrial function, oxidative stress and inflammation.

Interpretation of the obtained results has several limitations. In general, lack of accounting for confounding factors may explain the conflicting results in genetic association studies. Assessment of several candidate genes with minor effects if any, additive or in complex interactions, requires investigation on larger samples and still await for the development of adequate study approaches. Stratification according to sex and bodyweight could offer more conclusive results, and insulin, homocysteine and folate status evaluation or the use of more sensitive approaches to assess the hepatic and vascular burden could provide further insight. We analyzed effects according to the widely used dominant model, this approach, however, may be arguable, since there are also reports of over-dominant effects and significant changes observable only in recessive models. Nonetheless, our pathway based approach may represent an alternative to address variant interactions influencing disease progression by simple methodologies. Gene-gene and gene-environment interactions in general, and more importantly in these metabolic master genes need to be addressed, since due to frequency, involvement in common adult-onset disease etiology from the earliest stages of development, and modifiable nature of variant-associated effects by personalized interventions, novel information obtained could have also public health implications.

Conclusions

By various direct and indirect effects on metabolism, MTHFR may be considered a candidate gene for the MetS. Its common functional polymorphism rs1801133 may influence metabolic syndrome evolution, the age of onset of components - dysglicemia or dyslipidemia, and development of atherosclerotic complications but not NAFLD, in the local population of Caucasian origin. Simultaneous study with other polymorphisms, such as rs1801282 and rs8192678 of metabolic master genes possibly influenced by MTHFR suggests that besides simple independent additive effects within a polygenic system responsible for genetic predisposition, complex mitigating and aggravating variant interactions may exist, and the protective or predisposing outcome may depend on modifiable environmental factors.

Conflict of interest

None to declare.

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