

# Effect of the *MTHFR* 677C/T Polymorphism on Homocysteinemia in Response to Creatine Supplementation: A Case Study

M. PETR<sup>1</sup>, M. ŠTEFFL<sup>1</sup>, E. KOHLÍKOVÁ<sup>1</sup>

<sup>1</sup>Department of Physiology and Biochemistry, Faculty of Physical Education and Sport, Charles University in Prague, Prague, Czech Republic

Received March 19, 2013

Accepted May 2, 2013

On-line July 17, 2013

## Summary

Creatine (Cr) is recommended as a dietary supplement especially for athletes but its therapeutic potential is also discussed. It is assumed that human body uses Cr for the formation of phosphocreatine, which is necessary for muscular work as a source of energy. Production of Cr in a body is closely connected to methionine cycle where guanidinoacetate (GAA) is in a final step methylated from S-adenosylmethionine (SAM). Increased availability of SAM for phosphatidylcholine (PC) and sarcosine synthesis can potentially stimulate endogenous production of betaine a thus methylation of homocysteine (HCy) to form methionine. Our subject who was methylenetetrahydrofolate reductase (*MTHFR*) 677TT homozygote lowered plasma HCy from 33.3  $\mu\text{mol/l}$  to 17.1  $\mu\text{mol/l}$  following one-month Cr supplementation (5 g/day) opposite to 677CC and CT genotypes whose HCy levels tended to increase (but still in normal ranges). We suppose that Cr supplementation stimulates pathways leading to production of sarcosine which can serve to regenerate tetrahydrofolate (THF) to form 5,10-methylene-THF. This could potentially increase *MTHFR* enzyme activity which may later result in increased HCy methylation. Cr supplementation significantly effects metabolism of one carbon unit and potentially lower body's demands for methyl groups. This could be beneficial as in the case of reduced enzyme activity such as *MTHFR* 677C/T polymorphism.

## Key words

Creatine (Cr) • Homocysteine (HCy) • Supplementation • *MTHFR* gene • 677C/T

## Corresponding author

Miroslav Petr, Department of Physiology and Biochemistry, Faculty of Physical Education and Sport, Charles University

in Prague, José Martího, 162 52 Prague 6, Czech Republic.  
E-mail: petr@ftvs.cuni.cz

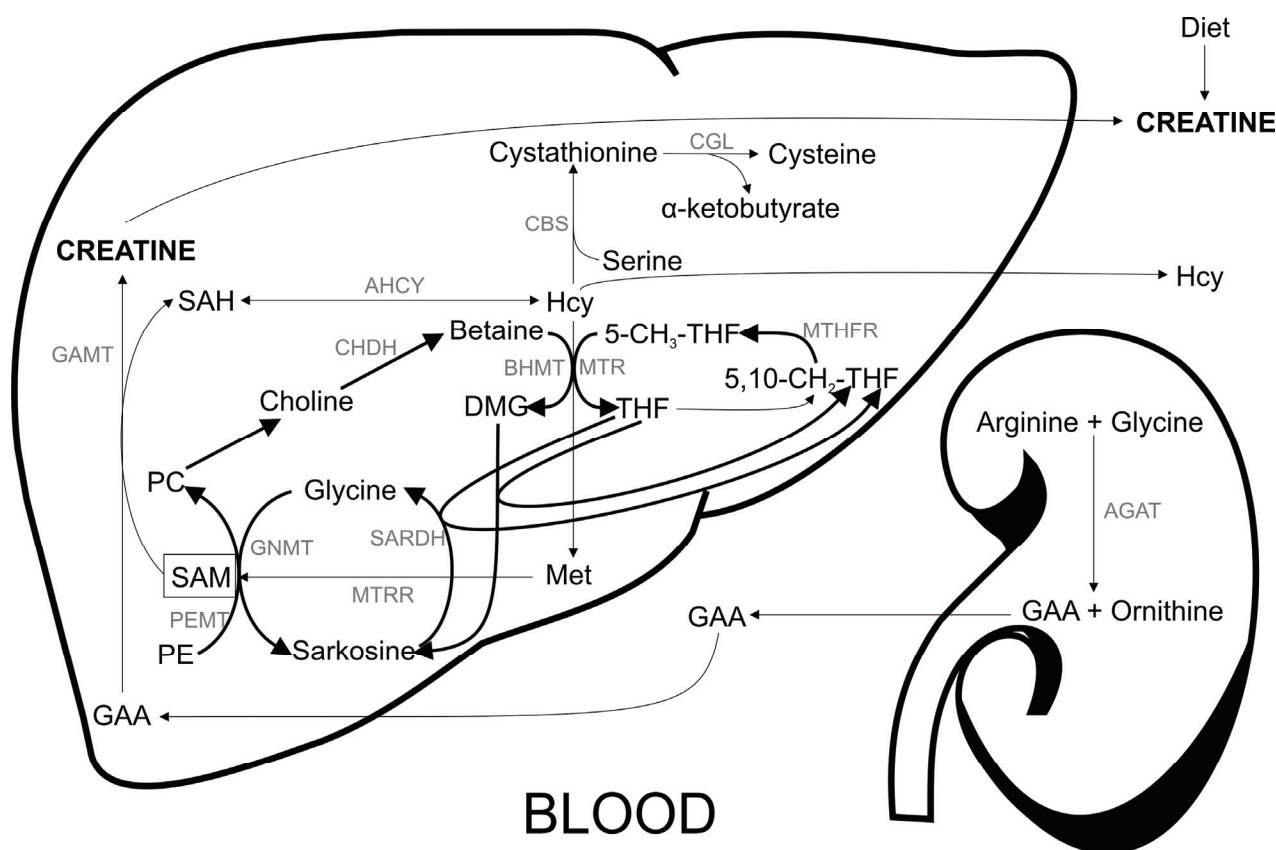
## Introduction

Creatine (Cr) supplementation has been widely used in athletes to support recovery in short term bouts of high-intensity exercise enabling more effective training and performance. Beneficial effect of Cr supplementation in young, healthy males is the enhanced muscle fiber size, strength and increased lean body mass (Greenhaff *et al.* 1994, Kreider *et al.* 1998). The greatest improvements in performance have been found in activities, which possibly stress the phosphocreatine (PCr) system such as series of high-power output exercises (Terjung *et al.* 2000). Studies have shown improved anaerobic exercise performance (Law *et al.* 2009), and specific performance in many sports such as fin swimming (Juhász *et al.* 2009), swimming, all-out cycling, sprinting, repeated jumping, and resistance training (Juhn and Tarnopolsky 1998).

The potential therapeutic value of Cr supplementation has recently been investigated with respect to various neurodegenerative disorders which include; Alzheimer's, Parkinson's, amyotrophic lateral sclerosis, and Huntington's disease patients (Adhietty and Beal 2008). In patients with diseases that result in atrophy or muscle fatigue secondary to impaired energy production there have shown beneficial effects from Cr supplementation. Cr supplementation has also been investigated in various neuromuscular diseases including mitochondrial cytopathies, neuropathic disorders, dystrophies, congenital myopathies, and inflammatory myopathies (Tarnopolsky and Martin 1999).

The daily turnover of Cr (1-2 g/ day) is replaced either through dietary Cr intake (from animal-derived tissues such as meat) or through endogenous synthesis in the liver from amino acid precursors (arginine, glycine and methionine). At the same time and approximately the rate (2 g/day), Cr is broken down to creatinine and excreted to the urine (Stead *et al.* 2006). The first step of Cr synthesis is the formation of guanidinoacetate (GAA) and ornithine in a reaction catalyzed by glycine amidinotransferase (AGAT), GAA can then be methylated on the original glycine nitrogen using *S*-adenosylmethionine (SAM) as the methyl donor. This reaction yields Cr and *S*-adenosylhomocysteine (SAH) and is catalyzed by the enzyme guanidinoacetate *N*-methyltransferase (GAMT) (Wyss and Kaddurah-Daouk 2000). The rate limiting step in Cr synthesis is the formation of guanidinoacetate by AGAT and it has been

shown that Cr supplementation down-regulates AGAT expression (Guthmiller *et al.* 1994). An increase in serum levels of Cr resulted in a decrease in AGAT enzyme activity, enzyme level, and mRNA expression in rat kidney (McGuire *et al.* 1984). On the other hand, the growth hormone up-regulates AGAT expression in rats (Guthmiller *et al.* 1994). AGAT is highly active in the kidneys, whereas GAMT is highly active in the liver. Therefore it is suggested that GAA is synthesized primarily in the kidney and then transported to the liver where it is methylated to form Cr (Fig. 1) (Wyss and Kaddurah-Daouk 2000). In rats, Van Pilsum *et al.* (1972) documented high activity of AGAT apart from kidney in pancreas, brain, spleen, and testes. In a study Edison *et al.* (2007) authors suggested that renal GAA production in a human may account only for about 20 % of total GAA synthesis.



**Fig. 1.** Biochemical pathways related to Cr metabolism and methionine cycle. Creatine supplementation leads to inhibition of its endogenous synthesis. Simultaneously PEMT and GNMT activity is stimulated leading to produce choline, betaine and sarcosine respectively. Production and degradation of sarcosine utilize THF to form 5,10-methylene-THF stimulating MTHFR activity (bold arrows). GAA, Guanidinoacetate; AGAT, Glycine amidinotransferase; SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine; GAMT, guanidinoacetate *N*-methyltransferase; PEMT, Phosphatidylethanolamine *N*-methyltransferase; GNMT, Glycine *N*-methyltransferase; PC, Phosphatidylcholine; MTR, Methionine synthase; MTRR, Methionine synthase reductase; MTHFR, Methylene tetrahydrofolate reductase; BHMT, Betaine homocysteine methyltransferase; CHDH, Choline dehydrogenase; THF, Tetrahydrofolate; SARDH, Sarcosine dehydrogenase; DMG, Dimethylglycine; Met, Methionine; AHCY, *S*-adenosylhomocysteine hydrolase.

Cr metabolism disorders have so far been described at the level of two synthetic steps, GAMT and AGAT, and at the level of the Cr transporter 1 (CrT1). The most common GAMT and AGAT deficiency symptoms and signs are delayed language development, learning disorders, autistic behavior, epileptic seizures, and movement disorders (Gordon 2010). GAMT and AGAT deficiency are treatable by oral Cr supplementation, but patients with Cr transporter deficiency do not respond to this type of treatment (Evangelidou *et al.* 2009). Neither nine months of L-arginine supplementation did not showed effectiveness in the four patients affected with Cr transporter deficiency (Fons *et al.* 2008).

In a human body, more than 90 % of SAM is used for methylation reactions by at least 50 different methyltransferases (Stead *et al.* 2006). There are three major methyltransferases playing an important role in the generation of homocysteine (HCy) and regulation of methyl group metabolism; except for GAMT they include phosphatidylethanolamine N-methyltransferase (PEMT), and glycine N-methyltransferase (GNMT) (Mudd *et al.* 2007) (Fig. 1). Production of Cr *via* GAMT and phosphatidylcholine (PC) *via* PEMT is considered to be the largest consumer of methyl groups derived from SAM. Stead *et al.* (2006) suggests opposite of original investment where GAMT has been proposed to consume up to 70 % of methyl groups (Mudd *et al.* 1980), and considers PEMT to be the primary consumer of methyl groups, having the greatest impact on HCy levels. It was found that *PEMT* *-/-* mice have lower choline pools in liver despite being fed sufficient or supplemental amounts of dietary choline (Zhu *et al.* 2003). When *PEMT* is deleted in mice, plasma HCy concentrations fall 50 % and, when it is over expressed, plasma HCy concentrations increase 40 %, demonstrating that PEMT activity is a very major consumer of SAM (and thereby a producer of HCy) (Jacobs *et al.* 2005). Furthermore, methylation of macromolecules such as DNA, RNA, histones, and other proteins play critical roles in cellular metabolism. Therefore, the level of SAM must be carefully regulated to maintain cellular homeostasis (Luka *et al.* 2009).

The serum concentration of HCy is positively associated with the risk of ischemic heart disease, deep vein thrombosis and pulmonary embolism, and stroke (Boushey *et al.* 1995). A meta-analysis by Boushey *et al.* (1995) of 27 studies showed that HCy was an independent, graded risk factor for atherosclerotic disease. Total HCy

(tHCy) measured in blood is usually the sum of free (reduced) HCy and protein-bound HCy (Brosnan *et al.* 2004). There are two methylation pathways to form methionine from HCy, both of which result in lowering HCy concentrations (Olthof *et al.* 2003). In the first, vitamins B<sub>12</sub> and folic acid are involved in a reaction catalyzed by methionine synthase (MTR) (Weisberg *et al.* 2001). Deficiencies of these vitamins can result in elevated plasma HCy concentrations (Bailey *et al.* 2002, He *et al.* 2010). Also single nucleotide polymorphisms in genes coding enzymes involved in this pathway can result in elevated HCy in blood. Examples are substitutions as *MTR* 2756A/G (Barbosa *et al.* 2008), methionine synthase reductase (*MTRR* 66A/G) (Naushad *et al.* 2008), methylenetetrahydrofolate reductase (*MTHFR* 677C/T and 1298A/C) (Weisberg *et al.* 2001), or methylenetetrahydrofolate dehydrogenase (*MTHFD1* 1958G/A) (Brody *et al.* 2002). The most profound effect on HCy levels in blood was found in *MTHFR* 677C/T and/or in *MTR* 2756A/G (Barbosa *et al.* 2008). The 677C/T variant which leads to the substitution of Ala-222 by valine produces MTHFR enzyme with reduced activity, resulting in an elevation of serum HCy concentrations of about 20 % (Brattstrom *et al.* 1998). The 677C/T polymorphism is surprisingly common, with about 10 % of people in the population being homozygous affected (TT), 47 % homozygous unaffected (CC), and 43 % heterozygotes (CT) (Brattstrom *et al.* 1998). In the Czech population similar frequencies were elicited (CC – 41 %, CT – 49 %, TT – 10 %) (Vesela *et al.* 2005).

An association with hyperhomocysteinemia was also found in *MTHFR* 1298A/C polymorphism (Barbosa *et al.* 2008), although its contribution seems to be lesser compared to 677C/T (Bailey *et al.* 2002, Barbosa *et al.* 2008). In additional studies, the activity of enzyme produced by the heterozygotes for both mutations was lower compared to individuals who carried only 677T/T (Chango *et al.* 2000). The genetic influence of the *MTHFR* polymorphism on HCy levels is attenuated in females in premenopausal age and is not significant in subjects who exhibit serum levels of folate and/or vitamin B<sub>12</sub> above the 50th percentile of distribution in the general population (Cortese and Motti 2001). A decrease in serum HCy of 3 mmol/l (achievable by daily intake of about 0.8 mg folic acid) should reduce the risk of ischemic heart disease by 16 %, deep vein thrombosis by 25 % and stroke by 24 % (Wald *et al.* 2002).

In the second pathway, the methylation of HCy to form methionine is catalyzed by betaine homocysteine

methyltransferase (BHMT) which is in addition to choline dehydrogenase (CHDH), and PEMT the most important enzyme in choline metabolism (da Costa *et al.* 2006). Betaine, once formed from choline *via* CHDH, donates its methyl group to Hcy *via* BHMT to form methionine. Administration of betaine can lower plasma Hcy concentrations (Steenge *et al.* 2003). Activity of BHMT is increased during methionine excess and plasma betaine was shown to be a strong determinant after methionine increase in tHcy in subjects not supplemented with B-vitamins (Holm *et al.* 2004). On the other hand, neither a common variant 742G/A, nor other variants in *BHMT* gene seem to play a significant role in plasma Hcy (Heil *et al.* 2000, Morin *et al.* 2003).

The aim of the present post-hoc analysis is to evaluate homocysteinemia following Cr supplementation in relation to *MTHFR* 677C/T genotype.

## Methods

### Subjects

This is a secondary analysis of data from 11 athletes participating in our previous study (Navratil *et al.* 2010) who provided DNA from buccal cells. Of those participants, 10 submitted DNA testing. All subjects were young, aged 24-28 years old, healthy, physically active persons, dealing with sportive activities (ice hockey, football, horsemanship, and athletics) on a professional level. Subjects' height, weight, and body composition *via* Bioelectrical Impedance Analysis (Multi-frequency analyzer In Body 3.0, Korea) was measured. All 10 men were Caucasian. Subject characteristics are shown in Table 1. Written informed consent was obtained from all subjects under protocols approved by the Institutional Ethics Committee of the Charles University of Faculty of Physical Education and Sport.

**Table 1.** Descriptive characteristics of 10 exercise-trained men.

Variable	Value
Age (y)	24.6±2.1
Height (cm)	181.7±4.8
Weight (kg)	82.8±11.0
Body fat (%)	12.0±2.9
BMI ( $\text{kg}\cdot\text{m}^{-2}$ )	25.0±2.4
Fat Free Mass (kg)	75.6±8.0

Values are means ± SD.

### Intervention

As previously published, all subjects ingested 5 g of CR-monohydrate (Plutino, Czech Republic) a day, diluted in tepid water. These doses were administered every morning (at about 8 a.m.) for 30 days. Participants were not allowed to consume any other supplement, especially those containing vitamin B and folic acid, and they were advised to maintain their usual dietary habits and physical activity during the study.

### Biochemical assays

Fasting blood and urine samples were collected at baseline and the next morning after completion 30-day Cr supplementation and further analyzed for several metabolites including Hcy as described previously (Navratil *et al.* 2010, Petr *et al.* 2011).

### Genotyping

Genomic DNA was isolated from buccal cells collected with cheek brushes (Whatman, USA). Samples were lysed and DNA was stabilized with DNA Extract All Reagents Kit (Applied Biosystems, USA) according to the manufacturer's protocol. Samples (5 µl) were genotyped according to the manufacturer's protocol on an Illumina BeadStation 500G Golden Gate genotyping platform using a custom panel (GS0011351-OPA) of 384 candidate single-nucleotide polymorphisms. From the whole set the genotypes for the *MTHFR* C677T polymorphism (rs1801133) were extracted.

## Results

Of 10 subjects, 9 individuals were carrying 677CC+CT, and 1 individual the 677TT genotype. Pre-test levels of plasma Hcy were normal among those carrying 677CC+CT genotype (6.3±1.3 µmol/l), but strongly elevated in 677TT carrier (33.2 µmol/l). After 30-day Cr supplementation individuals with 677CC+CT genotype mildly elevated Hcy levels, but completely different response was registered in 677TT carrier who lowered Hcy almost to normal levels (Table 2).

## Discussion

We found an individual response of plasma Hcy to Cr supplementation which was associated with genotypes in *MTHFR* 677C/T polymorphism. Our results cannot be considered as statistically significant due to the low number of subjects and the presence of just one TT

carrier. In spite of that, we assume our outcomes to be interesting, as they should contribute to better

understanding how supplemented Cr can affect methyl donor balance and consequent plasma Hcy levels.

**Table 2.** Pre-test and post-test Hcy levels in different *MTHFR* 677C/T genotypes.

	677CC (n=4)	677CT (n=5)	677TT (n=1)	677CC+CT
<i>Hcy</i> ( $\mu\text{mol/l}$ )				
<i>pre-test</i>	5.9 $\pm$ 1.3	6.6 $\pm$ 1.3	33.2	6.3 $\pm$ 1.3
<i>post-test</i>	9.9 $\pm$ 2.9	11.6 $\pm$ 3.3	17.1	10.9 $\pm$ 3.2

Values are means  $\pm$  SD.

A meta-analysis of published cohort studies states that hyperhomocysteinemia moderately increases the risk of a first cardiovascular event, regardless of age and follow-up duration (Bautista *et al.* 2002). In homocystinuria, which is a rare inherited disorder (most often due to cystathionine  $\beta$ -synthase deficiency, which occurs in 1 from 200.000 people), plasma Hcy levels are markedly elevated ( $>50 \mu\text{mol/l}$ ; normal range, 5 to 15  $\mu\text{mol/l}$ ), and patients have severe, widespread vascular disease (Bellamy *et al.* 1998). In the general population, mild to moderate elevations in plasma Hcy (15 to 35  $\mu\text{mol/l}$ ) are common and may occur due to inherited enzyme variants and/or a relative deficiency of folate, vitamin B<sub>12</sub>, or vitamin B<sub>6</sub>, which are required for the normal metabolism of Hcy (Ubbink *et al.* 1993).

Our subjects with CC and CT genotypes had pre-test Hcy concentration in normal ranges 6.1 $\pm$ 1.3  $\mu\text{mol/l}$  with milder individual differences. The only carrier of TT genotype had elevated Hcy (33.3  $\mu\text{mol/l}$ ). After 30-day Cr supplementation all CC and CT carriers increased plasma Hcy to 10.9 $\pm$ 3.2  $\mu\text{mol/l}$  opposite to TT carrier who significantly lowered Hcy levels to 17.1  $\mu\text{mol/l}$ . An interpretation of these changes is not simple to explain. Reliable description of addressed biochemical pathways should be supported by analysis of associated intermediates e.g. sarcosine in urine.

We assume that supplemented Cr inhibited the synthesis of endogenous Cr and thus increased availability of SAM for PC and sarcosine synthesis *via* PEMT and GNMT respectively. Concomitantly, the methylation of Hcy relied more on BHMT where methyl donor is betaine, stimulated by increased production of PC and choline *via* increased PEMT activity. Although PEMT is considered to be a major consumer of SAM and a producer of Hcy by some authors (Jacobs *et al.* 2005),

provided that PC and choline levels are sufficient from diet, Hcy methylation from betaine could be stimulated. Moreover, resulting dimethylglycine demands tetrahydrofolate (THF) for sarcosine formation *via* dimethylglycine-dehydrogenase. THF is also needed for glycine formation from sarcosine *via* sarcosine-dehydrogenase (SARDH). Sarcosine production can also be accelerated due to better availability of SAM for GNMT. Based on these facts TFH is dynamically converted to 5,10-methylene-THF (methylene tetrahydrofolate) stimulating MTHFR enzyme to 5-methyl-THF production which is later used for Hcy conversion to methionine *via* MTR. Higher availability of 5,10-methylene-THF could possibly effectively stimulate MTHFR even in 677TT carriers. This can have quite a strong effect on Hcy levels as we registered in our one 677TT carrier.

In fact, studies evaluated effects of Cr on Hcy levels are not consistent. Cr administration has been shown to decrease plasma tHcy by 25 % in rats (Stead *et al.* 2001) and by 10 % in humans, according to one report (Korzun 2004); in another report, this reduction in humans was not significant (Steenge *et al.* 2001). Opposite to these results, Cr supplementation (alone or in combination with L-arginine) was associated with an 11-20 % increase in Hcy concentration, which was not attributable to worsened renal function, providing evidence against an effect of Cr on decreasing methylation demand (Jahangir *et al.* 2009). In rats, plasma Hcy was increased up to 2 h after intense anaerobic exercise, but Cr supplementation decreased plasma Hcy independent on exercise intensity (Deminice *et al.* 2011). Recent study shows interesting results about the prevalence of hyperhomocysteinemia ( $>15 \mu\text{mol/l}$ ) in elite athletes which was 47 % compared to 17 % in

controls without any correlation between HCy and any of the other investigated variables, including plasma folate, vitamin B<sub>12</sub>, blood pressure, lactate dehydrogenase (LDH), creatine kinase (CrK), total and high-density lipoprotein (HDL) cholesterol and interleukin-6 (IL-6) (Borrione *et al.* 2008).

Our findings demonstrate an increase of HCy following Cr supplementation in *MTHFR* 677C allele carriers but an average and individual augmentation were all in normal ranges (<15 µmol/l). We suppose, when levels of dietary choline (PC) are sufficient to cover physical needs, a great part of choline from endogenous production of PC should be used to form betaine for further HCy methylation. Provided that choline from diet does not meet body demands, endogenous choline production (*via* PEMT) is very important. Under these conditions most probably less betaine can be produced to

methylyate HCy. Pathways following methylation with betaine seem to have a secondary methylation potential in regeneration of THF to form 5,10-methylene-THF. Cr supplementation significantly effects metabolism of one carbon unit and potentially lower body demands for methyl groups. This could be beneficial as in the case of reduced enzyme activity like *MTHFR* 677C/T polymorphism.

### Conflict of Interest

There is no conflict of interest.

### Acknowledgements

This study was supported by research grant No. MSM 002160864, SVV 2013-267603 and PRVOUK n. 38 founded by Faculty of Physical Education and Sport, Charles University in Prague.

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