# Endothelial Cell Dysfunction in Women with Cardiac Syndrome X and *MTHFR* C677T Mutation

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**Key words:** syndrome X, *MTHFR*, homocysteine

## Abstract

**Background:** The etiology of chest pain with normal epicardial coronary arteries (cardiac syndrome X) seems to be related to endothelial cell dysfunction. Multiple factors are implicated in the pathophysiology, including elevated levels of homocysteine in the blood. Mutations in the *MTHFR* gene are associated with elevated levels of homocysteine.

**Objectives:** To test whether abnormal homocysteine metabolism is associated with syndrome X.

**Methods:** Forty-two women with chest pain, positive stress test and normal coronary arteries (syndrome X) and 100 asymptomatic women (controls) were studied for the *C677T* mutation. Vitamin B12, folic acid, and plasma levels of homocysteine were also measured. Endothelial cell function was studied in 10 patients with syndrome X and homozygosity for *C677T* mutation, and in 10 matched healthy controls. Folic acid (5 mg daily) was prescribed to syndrome X patients after initial measurements of ECF. Following 13 weeks of treatment, ECF and blood tests were repeated and compared to baseline measurements.

**Results:** Homozygosity for *C677T* mutation was doubled in syndrome X vs. control (33%, 14/42 vs. 16%, 16/100, $P < 0.02$), and homocysteine levels were increased (9.16 ± 2.4 vs. 8.06 ± 2.6 µmol/L, $P = 0.02$). In the 10 homozygous patients, homocysteine levels decreased significantly after treatment with 5 mg/day folic acid (10 ± 3.3 vs. 5.4 ± 1.1 µmol/L, $P = 0.004$). Abnormal baseline ECF improved after treatment with folic acid: flow-mediated dilatation was greater (11.3 ± 7.9% vs. 0.7 ± 4.5%, $P < 0.002$), as was nitroglycerin-mediated dilatation (15.2 ± 9.0% vs. 5.6 ± 6.4%, $P < 0.003$). Frequency of chest pain episodes was significantly reduced after 13 weeks of folic acid treatment.

**Conclusion:** Our findings establish the association between the *C677T* mutation, endothelial cell dysfunction and cardiac syndrome X, and provide a novel and simple therapy for a subset of patients with syndrome X and homozygosity for the *C677T* mutation.

*IMAJ 2007;9:321–325*

The term syndrome X refers to patients with chest pain, positive stress test, and normal epicardial coronary arteries by angiography [1,2]. This syndrome is also known as microvascular angina [3] and is more common in women [4]. The etiology of the syndrome is uncertain but there is an emerging consensus that the pathophysiology is related to endothelial cell dysfunction, resulting in impaired coronary flow reserve [5]. The role of inflammation was recently suggested to play a role in the pathogenesis of syndrome X [6]. Patients with microvascular angina manifest endothelial cell dysfunction both in invasive and non-invasive tests [5,7].

Endothelial dysfunction is associated with hypertension, diabetes, smoking and hypercholesterolemia, and was also found to be associated with elevated levels of homocysteine [8]. Increased levels of homocysteine have been shown to induce endothelial dysfunction in healthy subjects after methionine loading [9]. Homocysteine may impair endothelium function by decreasing both the production of vasodilator substances and their bio-availability to smooth muscle cells [10].

Homozygosity for the *C677T* mutation in the $N_5,N_{10}$-Methyl enetetrahydrofolate reductase (*MTHFR*) gene is associated with elevated homocysteine [11], particularly in the presence of either folic acid or vitamin B12 deficiency [12].

This study was designed to test whether abnormal homocysteine metabolism is associated with syndrome X. To test for the association we examined the frequency of homozygosity for *C677T* mutation in the *MTHFR* gene in women with syndrome X. To further explore the potential mechanisms by which homocysteine can contribute to the etiology of syndrome X we tested endothelial cell function in women with syndrome X who are homozygous for the *C677T* mutation. After finding that the association between cardiac syndrome X and homozygosity for *C677T* does exist, we tested the effects of folic acid supplementation on homocysteine levels, endothelial cell function and well-being in these women.

## Patients and Methods

The study group consisted of 42 consecutive women with cardiac syndrome X. Patients were identified from catheterization laboratory logbooks. The diagnosis of cardiac syndrome X was based on

* The first two authors contributed equally to the study.

ECF = endothelial cell function

the presence of chest pain, positive stress test or effort-induced reversible perfusion defects found in thallium scintigraphy, and normal epicardial coronary arteries by angiography. Exclusion criteria included mild coronary disease, history of myocardial infarction or coronary intervention, valvular heart disease, cardiomyopathy, left ventricular hypertrophy, anemia and hyperthyroidism.

The control group comprised 100 female volunteers with no history of angina pectoris, myocardial infarction or other known cardiac disease. Volunteers were recruited from four factories and hospitals in northern Israel. The study was not officially published. None of the participants in the study was taking vitamin preparations. The study was approved by the institutional review board of the Lady Davis Carmel Medical Center and the Israel Ministry of Health. All participants agreed to participate in the study by signing an informed consent form. The investigation conforms to the principles outlined in the Declaration of Helsinki.

#### Study protocol

Genomic DNA was analyzed for the *MTHFR C677T* mutation. Serum levels of vitamin B12, folic acid, and plasma levels of homocysteine were measured in all patients and volunteers. Of the 42 patients tested 14 (33%) were found to be homozygouss for the *C677T* mutation. Ten homozygous patients and 10 subjects from the control group (matched for age, body mass index and smoking habits, and either hetrozygote or no mutation for *C667T* mutation) underwent forearm endothelial function studies. After the initial forearm endothelial function study, the 10 homozygous patients were treated with 5 mg folic acid per day for 13 weeks [13] (open-label non-randomized design). Those with B12 vitamin deficiency (< 200 pmol/L) (n=3) received, in addition, 1 mg vitamin B12 sublingual tablet per day. After 13 weeks of treatment all tests were repeated in these patients. In addition, patients were requested to document the number of chest pain episodes in the week prior to treatment and after 13 weeks of treatment and also to grade the pain severity of a typical pain episode on a scale of 1 to 10. Assessment was based on phone interviews by one of the researchers.

#### Blood sampling and genetic analysis

Twelve-hour fasting venous blood was collected in tubes containing disodium EDTA. Samples were promptly centrifuged (1500 rpm for 10 min) after collection and stored at -20°C. Plasma homocysteine levels were measured as total homocysteine by amino acid analyzer (Biochrom 20, Pharmacia, Canada) [14]. Blood analysis for vitamin B12 and folic acid levels was performed using a radioimmunoassay (Dual Count SPNB, DPC, USA). Glucose, cholesterol, triglycerides, creatinine and urea levels were analyzed using standard methods (Hitachi 747 Boehringer Mannheim, USA). Genomic DNA was extracted from venous blood cells using High Pure PCR Preparation Kit (Boehringer Mannheim). DNA was amplified by polymerase chain reaction using Taq DNA polymerase and suitable primers. For the *MTHFR* mutation, amplified fragments were restricted with Hinf 1, which recognizes the *C677T* mutation as described by Frosst et al. [15]. Following restriction, DNA fragments were separated by electrophoresis in 8% polyacrylamide gel.

#### Endothelial function studies

The method for non-invasive examination of endothelial function has been described in detail by Sorensen and colleagues [16]. High resolution ultrasound was used to measure changes in arterial diameter in response to increased flow (FMD), which is dependent on endothelial function, and glyceryl trinitate (NMD), an endothelial-independent vasodilator. The axillary artery was scanned using longitudinal views by B-mode ultrasound imaging using a 10 MHz linear array transducer. The center of the vessel was identified when the clearest images of the anterior and posterior walls of the artery were obtained, and the transmit zone was set to the level of the anterior wall. Depth and gain were optimized to identify the lumen-to-vessel wall interface and were kept constant until the final recording was obtained. Changes in diameter were assessed by M-mode during four consecutive cardiac cycles; measurements of the four cycles were averaged. Prior to initiation of the study, reproducibility of measurements was tested on four volunteers. The subject rested in the supine position for 15 minutes before the first scan and remained supine until the final scan was recorded. Scans were obtained at rest, during reactive hyperemia, after 10 minutes of rest, and 5 minutes after sublingual 400 µg glyceryl trinitrate spray. Reactive hyperemia was induced by release of a pneumatic tourniquet placed around the forearm at a pressure of 250 mmHg and maintained for 5 minutes. Changes in the vessel diameter were recorded 1 minute after cuff deflation. The distance between the point of ultrasound measurements and the ulnar olecranon process was measured for each subject so that testing after treatment would be performed at the same location. All studies were performed in a temperature-controlled room (20–25°C) after 12 hours of fasting and no cigarette smoking. The physician who performed all scans and measurements was unaware of the study design and previous test results. Endothelial function studies validation was done in previous work from our department [17].

#### Statistical analysis

Continuous variables were compared between the study and control groups using Student's *t*-test. Paired *t*-test was used for the analysis of treatment effect in the 10 treated patients. Dichotomous variables were compared using the chi-square test. The median test was used when the standard deviation was large. Logistic regression analysis was used to test the predictive value of homozygosity for the *C677T* mutation for the presence of syndrome X (actual group allocation serving as the dependent variable). Results are presented as mean ± standard deviation.

## Results

#### Genotype analysis

Of the 42 women with syndrome X, 14 (33%) were homozygous for the *MTHFR C677T* mutation [Table 1]. In the control group,

FMD = flow-mediated dilatation
NMD = nitrate-mediated dilatation

**Table 1.** Frequency of gene mutation in patients with syndrome X

| Type of genetic variant | Syndrome X (n=42) No. (%) | Control (n=100) No. (%) | *P** |
|---|---|---|---|
| Homozygosity for the *C677T* mutation in the *MTHFR* gene (TT) | 14 (33%) | 16 (16%) | < 0.02 |
| Heterozygosity for the *C677T* mutation in the *MTHFR* gene (CT) | 20 (47%) | 50 (50%) | NS |
| No mutation (CC) | 8 (19%) | 34 (34%) | 0.07 |

**P* was determined by Pearson's chi-square test.

**Table 2.** Epidemiological, clinical and laboratory chracteristics of the homozygote patients and matched controls

| | Patients (n=10) | Controls (n=10) | *P* |
|---|---|---|---|
| Age (yrs) | | | |
| Mean ± SD | 52 ± 9.7 | 53 ± 9.2 | NS |
| Range | 38–70 | 40–70 | |
| Menopause (%) | 60% | 60% | NS |
| Hormone replacement therapy | 20% | 10% | NS |
| BMI (kg/m$^2$) | | | |
| Mean ± SD | 26.9 ± 3.9 | 26.2 ± 4.3 | NS |
| Glucose (mg/dl) | 114 ± 78 | 108 ± 66 | NS |
| Total cholesterol (mg/dl) | 211 ± 26 | 215 ± 51 | NS |
| HDL-cholesterol (mg/dl) | 57 ± 12 | 54 ± 12 | NS |
| B12 (pmol/L) | 227 ± 93 | 325 ± 151 | NS |
| Folic acid (nmol/L) | 21 ± 13 | 29 ± 13 | NS |
| Homocystein (µmol/L) | | | |
| Mean | 10 ± 3.3 | 6.2 ± 1.2 | 0.006 |
| Median | 8.95 | 6.2 | |

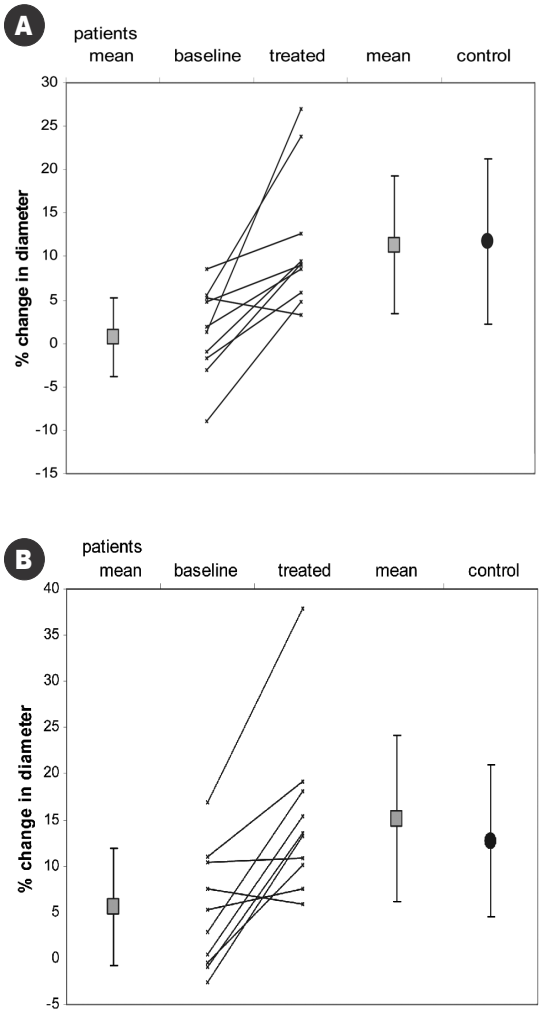


**Figure 1. [A]** Flow-mediated endothelial function (FMD) in syndrome X female patients at baseline and after 13 weeks of treatment. Mean of the patient group is presented (gray squares). Mean FMD of controls is shown as reference (black circle). Normalization of patient's FMD is noted after folic acid treatment. **[B]** Nitrate-mediated endothelial function (NMD) in syndrome X female patients at baseline and after 13 weeks of treatment. Mean of the patient group is presented (gray squares). Mean NMD of controls is shown as reference (black circle). Normalization of patient's NMD is noted after folic acid treatment.

16 of the 100 subjects (16%) were homozygous for the mutation. Using a logistic regression model with group allocation as the dependent variable, homozygosity for the *C677T* mutation was a significant predictor for syndrome X (odds ratio 2.62, 95% confidence interval 1.14–6.05). Patients who were homozygous for the *C677T* mutation tended to be younger when compared to patients without the mutation (52.7 ± 9 vs. 58.2 ± 9 years old, $P = 0.07$).

### Homocysteine, folate and B12 levels

Homocysteine levels were significantly increased in the syndrome X group when compared to the control group (9.16 ± 2.4 vs. 8.06 ± 2.6 µmol/L, $P = 0.02$). No significant difference was found between the patients and controls in folate levels. B12 levels were significantly lower in patients in comparison to controls (249 ± 107 vs. 310 ± 199 pmol/L, $P < 0.02$).

### Endothelial cell function

There were no significant differences in epidemiological, clinical and laboratory parameters between the 10 patients and 10 control individuals studied [Table 2]. Only homocysteine levels were significantly higher in the 10 patients when compared to the 10 healthy volunteers in the control group (10 ± 3.3 vs. 6.2 ± 1.2 µmol/L, $P = 0.006$). Endothelial cell dysfunction was present in all syndrome X patients. Flow-mediated dilatation was reduced compared to the control group (0.7 ± 4.5% vs. 11.7 ± 9.5%, $P < 0.006$) as was nitrate-mediated dilatation (5.6 ± 6.4% vs. 12.7 ± 8.2%, $P < 0.05$).

### Effects of folic acid treatment

After treatment, patients' folic acid levels were significantly higher than baseline levels (50.6 ± 10.6 vs. 21.3 ± 13.4 nmol/L, $P = 0.0002$) and higher than those of the controls (50.6 ±10.6 vs. 28.8 ± 13 nmol/L, $P = 0.0006$). Homocysteine levels after treatment were significantly reduced as compared to baseline (5.4 ± 1.1 vs. 10 ± 3.3 µmol/L, $P = 0.004$). A significant improvement in

both FMD (11.3 ± 7.9% vs. 0.7 ± 4.5%, $P < 0.002$) and NMD (15.2 ± 9 vs. 5.6 ± 6.4%, $P < 0.003$) was observed after treatment [Figure 1]. After 13 weeks of folic acid treatment no difference in endothelial function was found between the treated patients and the control group [Figure 1]. The number of chest pain episodes was reduced from 6 ± 3 episodes/week prior to folic acid therapy to 3.4 ± 2 episodes/week after 13 weeks of therapy ($P < 0.001$). There was a tendency to reduced pain intensity, from 4.8 ± 2 to 3.7 ± 2 ($P = 0.09$).

## Discussion

The findings of this study imply an association between *C677T* mutation homozygosity, cardiac syndrome X and endothelial dysfunction. The number of patients studied is relatively low to establish a solid genetic association between syndrome X and *C677T* mutation [18], but the high frequency of the mutation in our well-defined group of patients does imply that the mutation is associated with syndrome X. Woo and co-workers [8] showed that elevated homocysteine levels can cause endothelial dysfunction. The connection between the *MTHFR* gene mutation and elevated homocysteine levels was confirmed in a meta-analysis analyzing data of 12,513 subjects [19].

In our study, homozygosity for the *MTHFR C677T* mutation was more frequent in cardiac syndrome X patients and was associated with significantly higher homocysteine levels. The prevalence of homozygosity for the *C677T* mutation in the healthy Israeli female population was reported to be 8% [20]. Frequency of the mutation in Israeli patients consecutively admitted to five medical centers was 10.4% [21]. The increased frequency of homozygosity in the current study group (33%) substantiates the association between this mutation and cardiac syndrome X.

The working hypothesis of this study was that patients with syndrome X who are homozygous for the *MTHFR C677T* mutation suffer from endothelial dysfunction due to increased homocysteine levels and that folic acid treatment can reduce homocysteine and could emend endothelial function.

For assessment of endothelial function we matched the control and patient groups for risk factors predisposing to endothelial cell dysfunction, yet both FMD and NMD were abnormal in the syndrome X-homozygous group, while those of the control group were in the normal range. This exceptional finding implies that endothelial dysfunction is associated with the *MTHFR* mutation and the resulting high homocysteine levels. Previous studies have shown that similar homocysteine levels induce endothelial dysfunction [22]. Homocysteine is known to decrease the production of vasodilators, such as prostacyclin and nitric oxide, and the availability of these vasodilators to vascular smooth muscle cells [10]. The former phenomenon can explain the reduced FMD, and the latter, the reduced NMD, in our patients. Forearm endothelial cell dysfunction in patients with syndrome X and reversible cardiac perfusion defect was reported by Masci et al. [23].

Treatment with folic acid improved endothelial function considerably and reduced homocysteine levels. Normal endothelial function was restored, and the difference between the control and patient groups was abolished. Improvement of FMD and NMD with folic acid could reflect augmentation of endothelial NO synthase, and antioxidant effect [24]. Previous studies of folic acid treatment in hypercholesterolemia and fat loading observed that folic acid treatment improved FMD, but had no effect on NMD [25]. Similar to our findings, folic acid prevented both NO synthase dysfunction induced by continuous nitroglycerin and nitrate tolerance in the arterial circulation of healthy volunteers [24]. The fact that both endothelial-dependent (FMD) and endothelial-independent (NMD) vascular responses were impaired in our patients indicates that NO availability was reduced in these patients. The cause of reduced NO availability is unknown. Since both FMD and NMD were improved with folic acid treatment and the reduced homocysteine levels, we suggest that homocysteine has a role in the pathogenesis of patients with syndrome X and homozygosity for the *C677T* mutation. Based on our findings, the concept of microvascular dysfunction in patients with syndrome X, specifically those with the *MTHFR* mutation, could be defined as a syndrome of "physiologically frozen" arteries with both endothelial-dependent and independent dysfunction. Increased vascular stiffness in syndrome X patients was also reported by Arroyo-Espliguero et al. [6].

The management of patients with syndrome X is often frustrating due to the multiple pathogenic mechanisms and etiologies of this syndrome. We provide an additional therapeutic option for syndrome X patients that led to fewer symptoms and improvement in endothelial function in our patients. Folic acid, based on our observation, is probably efficient in women with syndrome X and *C677T* homozygosity.

## Conclusions

The findings of the current study provide a novel diagnostic and therapeutic approach to a subset of patients with cardiac syndrome X, who currently present a therapeutic challenge [1]. Folic acid supplement can improve endothelial function in these patients.

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NO = nitric oxide

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*Youth is the first victim of war – the first fruit of peace. It takes 20 years or more of peace to make a man; it takes only 20 seconds of war to destroy him*

Boudewijn I (1934-1993), King of Belgium

## Capsule

### Natural sunblock

Before the health hazards of ultraviolet (UV) light exposure were fully appreciated, sun worshippers applied lotions hoping to tan rather than burn. Skin tanning results from the production of the pigment melanin, which absorbs UV radiation and can partially protect cells from the UV-induced DNA damage that can ultimately cause skin cancer. Without melanin, cells are highly susceptible to sunlight; sunburn is the body's response to this damage. Cui et al. show that the tumor suppressor p53, which functions as a transcription factor and is one of the most intensely studied proteins in biology, plays a crucial role in UV-induced melanin production. Studying p53-deficient mice as well as normal human skin samples, they found that UV light activates p53 in skin keratinocytes (the outermost cells) and that p53 activates the gene encoding pro-opiomelanocortin (POMC). The POMC protein is then cut in several places to generate peptides, including alpha-melanocyte-stimulating hormone, which stimulates melanocytes to produce melanin. Interestingly, POMC proteolysis also generates the opioid peptide beta-endorphin, which the authors speculate might contribute to sun-seeking behavior in humans.

*Cell* 2007;128:853

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