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Folate/homocysteine phenotypes and *MTHFR* 677C>T genotypes are associated with serum levels of monocyte chemoattractant protein-1

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Abstract Monocyte chemoattractant protein-1 (MCP-1) is a chemokine that recruits monocytes into the subendothelial cell layer in atherosclerotic lesions. Elevated homocysteine (hyper-homocysteinemia), which is usually associated with low-folate status, is a known risk factor for many pathologies with inflammatory etiologies. The present study was undertaken to examine whether there are associations between MCP-1 concentrations and folate/Hcy phenotype or methylenetetrahydrofolate reductase (*MTHFR*) 677C>T genotype in healthy young adults. In females, MCP-1 concentrations were positively correlated with Hcy and negatively correlated with both serum and red blood cell folate; female smokers and *MTHFR* 677T carriers had particularly elevated MCP-1 concentrations. Similar relationships were not seen in males. These findings may have implications for understanding the female predominance observed for a range of autoimmune diseases, such as systemic lupus erythematosus and rheumatoid arthritis.
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Introduction

Hyperhomocysteinemia (HHcy), defined by circulating blood levels of homocysteine (Hcy) ≥ 13 μmol/L, is associated with several pathologies including cardiovascular disease (CVD),

stroke [1], neural tube defects (NTDs) in offspring [2], certain cancers [3], and rheumatoid arthritis (RA) [4,5]. Hcy is also elevated in patients with systemic lupus erythematosus (SLE) [6], an inflammatory disease that mainly affects young women and is associated with high cardiovascular comorbidity.

Hcy concentrations are affected by the availability of B vitamins, especially folate. They are also influenced by functional polymorphisms in key enzymes in the folate/Hcy metabolic pathway that can affect the distribution of the intermediates in the pathway and lead to elevated Hcy. Of these polymorphisms, the *MTHFR* 677C>T polymorphism has the greatest quantitative effect, with the 677TT genotype being associated with relatively high Hcy concentrations, particularly when folate status is low [7,8].

Elevated Hcy may be indicative of an underlying dysregulation of folate/Hcy metabolism. Several of the pathologies that are associated with folate/Hcy dysregulation have inflammatory components. However, the relationship between folate/Hcy phenotypes and inflammatory mediators has not been fully elucidated. Chemokines, which promote directed migration of inflammatory cells, are important components of the pathogenesis of atherosclerosis. Monocyte chemoattractant protein-1 (MCP-1) is a chemokine, encoded by the *CCL2* gene [9], that recruits monocytes into the subendothelial cell layer in atherosclerotic lesions. MCP-1 is also important in the recruitment of macrophages and leukocytes into the glomeruli and interstitial spaces of the kidney, and is consequently a significant contributor to nephritis, a major cause of morbidity and mortality in SLE [10]. Additionally, this chemokine is highly expressed in serum, synovial tissue and synovial fluid in patients with RA, another inflammatory disease [11].

In vitro studies have shown a link between Hcy and MCP-1. Expression and secretion of MCP-1 and IL-8 are upregulated in human aortic endothelial cells following treatment with pathophysiological concentrations of Hcy, thereby altering endothelial cell function [12]. Recently, it has been shown that chronic "low-folate stress" increases MCP-1 synthesis in EA.hy 926 endothelial cells; cells grown in low-folate culture medium express MCP-1 mRNA and protein at higher concentrations than those grown in high-folate medium, independent of Hcy [13].

Several conditions associated with a high-Hcy/low-folate phenotype may have shared aspects of their underlying etiologies, such as changes in inflammatory mediators. In a study of the relationship between MCP-1 and Hcy in women with SLE and matched controls, MCP-1 concentrations were higher in patients, and were positively correlated with Hcy [14]. In a subsequent pilot study [15], non-significant trends towards associations between MCP-1 concentrations and both folate/Hcy phenotype and *MTHFR* 677C>T genotype were observed in pre-menopausal Caucasian women. That MCP-1 is also affected by age, sex, race, body mass index (BMI) and smoking status has variously been reported for the above studies and by others [16,17]. To date, there have been no studies of sufficient size to determine whether folate and *MTHFR* 677C>T genotype are significant determinants of MCP-1 concentrations in young, healthy males and females.

The study population presented here comprises healthy females and males between the ages of 20 and 26 years, in which folate/Hcy phenotype has been shown to be influenced by different genetic and lifestyle factors that act in a sex-specific manner [18]. This study population is therefore ideal for testing the hypothesis that there are biologically significant positive associations between MCP-1 and Hcy concentrations, and negative associations between MCP-1 and folate concentrations, and to assess whether such associations are restricted to, or more prominent in, reproductive-aged females compared to males of the same age group. The implications of the findings for elucidating etiologic aspects of autoimmune diseases, which are more common in women than men, are discussed.

Materials and methods

Study subjects

Study subjects were enrolled in the Young Hearts Project (YH), an ongoing longitudinal study that initially examined the prevalence of coronary risk factors in a sample of young people (aged 12 and 15) from Northern Ireland recruited between 1989 and 1990. All of the subjects in the original cohort were invited back to participate in a hospital-based screening visit between 1997 and 1999, when the subjects were between the ages of 20 and 26 years, at which time the blood samples (obtained after an overnight fast) and demographic/lifestyle data used in this report were collected. The participation rate for this phase of the study was 48.2%, with 250 males and 239 females. Compared to non-respondents, these subjects tended to be from families with higher socioeconomic status and to have lower BMI at the baseline examination [19]. Subjects were broadly classified as smokers or non-smokers, based on self-report at the time of follow-up. Ethical approvals were granted by the Research Ethics Committee of Queen's University Belfast, and all subjects provided written informed consent.

There were 57 *MTHFR* 677TT homozygotes for whom serum samples were available. The more numerous *MTHFR* 677CC homozygotes and CT heterozygotes were matched to the TT homozygotes by both sex and smoking status. Within each resulting genotype-phenotype sub-class, subjects were randomly selected using SAS version 9.1 to generate sets of 57 CC and 57 CT subjects, each of which had the same distribution of male and female smokers and non-smokers as in the TT set.

Biochemical parameters

Blood samples were obtained from fasted subjects for determination of biochemical parameters. tHcy concentrations were measured using high performance liquid chromatography [20]. Serum folate concentrations were assessed using time-resolved immunofluorescence on an AutoDelfia analyzer [21]. RBC folates were determined using a microbiological assay [22]. MCP-1 concentrations were measured in archived serum samples using a human MCP-1 ELISA kit (BD Biosciences) according to the manufacturer's instructions.

Genotyping

The DNA extraction method and *MTHFR* 677C>T genotypes have previously been reported [21]. *CCL-2* (-2518)A>G genotypes were generated from the archived DNA samples

using a previously reported method [15]. Briefly, genomic DNA was mixed with 0.5 μM each of forward (5′-TTCTTGA-CAGAGCAGAAGTGG-3′) and reverse (5′-GCCTTTGCATATATCA-GACAGTA-3′) primers, 50 nM each of "A"-allele probe (5′-6FAM-AGACAGCTATCACTT-3′-MGBNFQ) and "G"-allele probe (5′-VIC-AGACAGCTGTCACTTTC-3′-MGBNFQ) in 20 μL Taqman master mix (Applied Biosystems). PCR was performed with an initial incubation at 95 °C for 10 min, 60 cycles of denaturation at 95 °C for 15 s and the extension/5′ nuclease step at 57 °C for 30 s.

Statistical analyses

Statistical analyses were performed using SAS version 9.1 with type I error rate set at 0.05. MCP-1 concentration was log-transformed to better approximate normality in all analyses. Differences between subsets of the study subjects (e.g. males and females) in the distribution of categorical variables were assessed using chi-square analysis or the Fisher's exact test, and differences in continuous variables were assessed using the t-test. Simple linear regression analyses were performed with log MCP-1 concentration as the outcome measure. Results of log MCP-1 analyses were back-transformed to report results in original measurement units (pg/mL).

Results

A total of 75 men and 96 women were selected from the "Young Hearts" population [19] for this study. They com-prised equal numbers of each *MTHFR* 677C>T genotype class (*i.e.* CC, CT, and TT), matched by sex and smoking status. The mean age for men was 22.4 and for women it was 23.1 years. The mean MCP-1 concentration in males was 319.7 pg/mL and in females was 261.1 pg/mL (p=0.004). As MCP-1 concentrations and several other variables differed significantly between males and females, all analyses were performed separately by sex. The characteristics of the study subjects are summarized in Table 1.

MCP-1 concentrations in relation to age and lifestyle variables

MCP-1 concentrations were not significantly associated with age, vitamin use, or alcohol consumption in either females or males (Table 2). MCP-1 was positively associated with BMI in females (p=0.02), but not in males. The BMI variable explained approximately 5% of the variation in MCP-1 concentrations in females. Among females, smokers had higher MCP-1 concentrations than non-smokers, although this relationship did not reach statistical significance (276.9 vs. 246.2 pg/mL, p=0.08). MCP-1 concentrations were not associated with smoking status in males.

MCP-1 concentrations in relation to biochemical phenotypes

In females, Hcy concentrations were positively associated with MCP-1 (p=0.01) and explained approximately 6% of the variation in MCP-1 concentration. Further, among females,

Table 1 Subject characteristics, biochemical phenotypes, and genotypes.

Variables	Sex		p-value
	Males (*N*=75)	Females (*N*=96)	
Age [a]	22.4±1.6 (75)	23.1±1.6 (96)	0.01 [d]
BMI (kg/m²) [a]	24.0±3.3 (75)	24.0±4.3 (95)	0.90 [d]
Vitamins [b]			
Yes	58 (77.3)	73 (76.0)	
No	17 (22.7)	23 (24.0)	0.86 [e]
Alcohol [b]			
No	13 (17.3)	22 (23.2)	
Yes	62 (82.7)	73 (76.8)	0.45 [e]
Cigarettes [b]			
No	50 (67.6)	48 (50.0)	
Yes	24 (32.4)	48 (50.0)	0.06 [e]
MCP-1 (pg/mL) [c]	319.7 [265.3–371.8] (75)	261.1 [210.5–315.8] (96)	<0.001 [d]
Hcy (μmol/L) [a]	11.8±7.7 (75)	10.5±5.5 (94)	0.24 [d]
Serum folate (nmol/L) [a]	14.3±7.9 (71)	15.1±10.5 (91)	0.56 [d]
Red cell folate (nmol/L) [a]	321.0±140.5 (70)	272.4±156.1 (93)	0.04 [d]
CCL-2 (-2518)A>G [b]			
AA	39 (52.7)	46 (49.5)	0.70 [f]
AG	31 (41.9)	38 (40.9)	
GG	4 (5.4)	9 (9.7)	

[a] Mean±SD (*n*).
[b] Count (percentage).
[c] Mean [inter-quartile range] (*n*).
[d] *p*-value by *t*-test.
[e] *p*-value by chi-square.
[f] *p*-value by Fisher's exact test.

Table 2 Proportion of variation (R^2) in MCP-1 levels explained by subject characteristics, biochemical phenotypes, and genotypes, by sex.

Variables	Sex			
	Males ($N=75$)		Females ($N=96$)	
	Variable coefficient (SE)	R^2 (p-value)	Variable coefficient (SE)	R^2 (p-value)
Subject characteristics				
Age (years)	0.002 (0.008)	0.0007 (0.82)	0.001 (0.009)	0.0002 (0.90)
BMI (kg/m^2)	−0.002 (0.004)	0.005 (0.54)	0.008 (0.003)	0.06 (0.02)
Vitamins (no/yes)	−0.05 (0.03)	0.04 (0.08)	0.003 (0.03)	0.0001 (0.93)
Cigarettes (no/yes)	0.003 (0.03)	0.0002 (0.92)	0.05 (0.03)	0.03 (0.08)
Alcohol (no/yes)	−0.02 (0.03)	0.008 (0.44)	−0.04 (0.04)	0.01 (0.32)
Biochemical phenotypes				
Homocysteine (μmol/L)	−0.0006 (0.002)	0.002 (0.70)	0.006 (0.003)	0.06 (0.01)
Serum folate (nmol/L)	0.0005 (0.002)	0.001 (0.77)	−0.003 (0.001)	0.06 (0.02)
RBC folate (nmol/L)	0.00003 (0.00004)	0.007 (0.51)	−0.00009 (0.00004)	0.05 (0.03)
Genotypes				
MTHFR 677C>T				
CT	−0.02 (0.03)	0.02 (0.57)	0.06 (0.04)	0.04 (0.14)
TT	0.007 (0.03)		0.06 (0.04)	
T carriers	−0.008 (0.03)	0.001 (0.76)	0.06 (0.03)	0.04 (<0.05)
CCL-2 (-2518)A>G				
AG	0.01 (0.03)	0.004 (0.88)	0.01 (0.03)	0.01 (0.64)
GG	0.008 (0.06)		0.05 (0.05)	
G carriers	0.009 (0.02)	0.002 (0.71)	0.02 (0.03)	0.006 (0.45)

MCP-1 concentrations were inversely associated with serum folate ($p=0.02$) and RBC folate ($p=0.03$). Serum folate and RBC folate explained approximately 6% and 5%, respectively, of the variation in serum MCP-1 concentrations. No such associations between MCP-1 concentrations and Hcy, serum folate, or RBC folate were observed in males.

MCP-1 concentrations in relation to genotypes

Among females, *MTHFR* 677T carriers had significantly higher MCP-1 concentrations than CC homozygotes (273.8 vs. 237.5 pg/mL, $p<0.05$); the CT and TT genotype classes had similar concentrations (273.7 and 273.9 pg/mL, respectively). In contrast, among males, *MTHFR* 677T carriers had MCP-1 concentrations that were similar to those observed in CC homozygotes (317.7 vs. 323.6 pg/mL, $p=0.76$) (Tables 2 and 3). MCP-1 was not associated with *CCL-2* (-2518)A>G genotypes in either females or males (Table 3).

Discussion

Altered folate/Hcy metabolism and increased MCP-1 concentrations are both potentially pathologic aspects of many human diseases in which inflammation is an etiologic

Table 3 Mean MCP-1 level by sex and other covariates.

Variables	Mean MCP-1 level (pg/mL) [inter-quartile range]			
	Males	p-value[a]	Females	p-value[a]
Cigarettes				
No	317.0 [258.1−366.0]	0.92	246.2 [206.2−293.6]	0.08
Yes	319.0 [279.8−380.8]		276.9 [216.5−343.9]	
MTHFR 677C>T				
CC	323.6 [296.2−371.8]	0.57	237.5 [203.9−282.9]	0.14
CT	306.8 [257.3−349.7]		273.7 [214.0−331.0]	
TT	329.0 [258.1−394.2]		273.9 [226.8−308.0]	
T carriers	317.7 [257.3−366.0]	0.76	273.8 [216.2−326.1]	<0.05
CCL-2 (-2518)A>G				
AA	317.8 [279.9−364.9]	0.88	253.9 [204.5−308.9]	0.64
AG	325.0 [253.1−398.4]		263.1 [211.9−312.8]	
GG	321.1 [256.6−410.6]		291.1 [251.7−321.7]	
G carriers	324.6 [253.1−398.4]	0.71	267.4 [215.9−321.7]	0.45

[a] *p*-values taken from relevant linear regressions in Table 2.

feature. A study of women with SLE and matched controls found that in this autoimmune disease, characterized by systemic inflammation, MCP-1 concentration is positively associated with Hcy concentration [14]. In a recent small pilot study in healthy, pre-menopausal women [15], a trend towards a reciprocal relationship between MCP-1 concentration and folate concentration was observed, though the results reported did not reach statistical significance.

The above *in vivo* observations in healthy and inflamed subjects have their corollary in *in vitro* studies that demonstrate a link between chronic low-folate stress and increased MCP-1 synthesis [13,23]. In the absence of any inflammatory stimulus, cells grown in low-folate culture medium have higher MCP-1 mRNA and protein expression than folate-replete cells [13]. This finding appears to be mediated through a stress response driven, at least in part, by increased cellular levels of p38 acting on the *CCL-2* promoter [23]. In the presence of an inflammatory stimulus, such as the cytokine TNF-α, MCP-1 transcription is massively induced via an NF-κB-dependent mechanism; however, the transcriptional and synthetic advantage of MCP-1 seen in the absence of cytokine stimulation is retained. Taken together, these *in vitro* observations suggest that, *in vivo*, individuals with a low-folate, high-Hcy phenotype would have elevated MCP-1 concentrations relative to their folate-replete peers, regardless of health status. The results reported here support this hypothesis in a population of healthy young women, but not in males.

Given the known responsiveness of the *CCL-2* gene to overt inflammatory stimuli, it might be expected that factors that have been linked to sub-clinical inflammation would also be associated with elevated MCP-1 concentrations. This study supports such an association between smoking and high MCP-1 concentrations in women only. In addition, elevated BMI, which is also often accompanied by sub-clinical inflammation, was associated with increased MCP-1 concentrations in women.

There was no association between *CCL-2* (-2518)A>G genotype and MCP-1 concentrations in this study population. The *CCL-2* (-2518)A>G polymorphism, located in the promoter region of the gene that encodes MCP-1, modifies the degree to which MCP-1 expression is induced by proinflammatory stimuli [24]. The (-2518)G allele is associated with increased MCP-1 transcription mediated by interleukin-1, and consequently with higher circulating concentrations of MCP-1 protein [24,25]. As the subjects in the present study were healthy and without known overt transient or chronic inflammatory conditions, this result is not surprising.

The relationships observed between MCP-1 and folate, Hcy, and *MTHFR* 677C>T genotype in females are consistent with the published *in vitro* studies. HHcy is associated with an increased secretion of chemokines, including MCP-1, that can be reversed by low-dose folic acid treatment [26]. This effect is compatible with mechanisms involving the Hcy-lowering effects of folic acid, the augmentation of cellular folate status per se, or a combination of each. We hypothesized that a relationship would exist between *MTHFR* 677C>T genotype and MCP-1 concentration based on (i) the effect that the functional polymorphism of the enzyme specified by the T allele has on Hcy and folate derivative distribution, and (ii) the positive and reciprocal quantitative relationships that these biochemical variables have with MCP-1. Specifically, as T carriers have altered folate and Hcy concentrations, we

speculated that they would have higher MCP-1 concentrations as was reported here for female T allele carriers.

In this study, the association between folate/Hcy phenotype and MCP-1 concentrations is restricted to females. Previous studies on this population have shown that Hcy and folate concentrations are influenced by different genotypic and phenotypic factors in a sex-specific manner [18], so the absence of analogous findings regarding folate/Hcy phenotype and MCP-1 in males has a precedent. This observation suggests that the mechanistic link between folate/Hcy metabolism and MCP-1 synthesis might involve sex hormones. Whether or not this is the case, the novel associations observed here between folate, Hcy and MCP-1 may have implications for understanding the complex etiology of autoimmune inflammatory diseases such as SLE and RA that are more common in females than in males. These findings could also have important implications for pregnancy related pathologies, including folate preventable birth defects such as spina bifida. MCP-1 is produced by endometrial tissue in very early pregnancy [27], and the *CCL-2* (-2518)A>G polymorphism has been associated with the risk of spina bifida [28].

In conclusion, several pathologies associated with altered folate/Hcy metabolism are inflammatory in nature, including SLE, RA, and cardiovascular disease. The associations of serum folate, RBC folate, and *MTHFR* 677C>T genotype with MCP-1 strongly suggest that MCP-1-mediated inflammation may be a causative or contributing factor to pathologies in which folate/Hcy dysregulation is an underlying feature.

Acknowledgments

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