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Polymorphisms in ABCG5 and ABCG8 Transporters and Plasma Cholesterol Levels

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Summary

A*BCG5* and *ABCG8* transporters play an important role in the absorption and excretion of sterols. Missence polymorphisms (Gln604Glu in the *ABCG5* and Asp19His, Tyr54Cys, Thr400Lys, and Ala632Val in the *ABCG8*) in these genes have been described**.** In 131 males and 154 females whose dietary composition markedly changed and lipid parameters decreased over an 8-year follow-up study (total cholesterol decreased from 6.21±1.31 mmol/l in 1988 to 5.43±1.06 mmol/l in 1996), these polymorphisms were investigated using PCR. Plasma lipid levels and changes in plasma lipid levels were independent of the Gln604Glu polymorphism in *ABCG5* and Asp19His and the Ala632Val polymorphisms in *ABCG8*. The Tyr54Cys polymorphism influenced the degree of reduction in total plasma cholesterol (Δ –0.49 mmol/l in Tyr54 homozygotes vs. Δ +0.12 mmol/l in Cys54 homozygotes, $p<0.04$) and LDL-cholesterol (Δ –0.57 mmol/l in Tyr54 homozygotes vs. Δ +0.04 mmol/l in Cys54 homozygotes, $p<0.03$) levels between 1988 and 1996 in females, but not in males. Male Thr400 homozygotes exhibited a greater decrease in total cholesterol (Δ –0.90 mmol/l vs. Δ –0.30 mmol/l, $p<0.02$) and LDL-cholesterol (Δ –0.62 mmol/l vs. Δ –0.19 mmol/l, $p<0.04$) than Lys400 carriers. No such association was observed in females. We conclude that Tyr54Cys and Thr400Lys variations in the *ABCG8* gene may play a role in the genetic determination of plasma cholesterol levels and could possibly influence the gender-specific response of plasma cholesterol levels after dietary changes. These polymorphisms are of potential interest as genetic variants that may influence the lipid profile.

Key words
Lipids • Polymorphism • Cholesterol • Diet • ABCG5 • ABCG8

Introduction

Coronary artery disease (CAD) is the most common cause of death in all industrialized countries, and high plasma lipid levels (cholesterol and triglycerides) are one of the most important risk factors of CAD.

Recently, two adjacent ATP-binding cassette

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(ABC) transporters, ABCG5 and ABCG8, have been described (Berge *et al.* 2000, Lee *et al.* 2001). The genes are located in head-to-head orientation on chromosome 2 and the proteins are expressed exclusively in the liver and intestine. Mutations in the *ABCG5* and *ABCG8* genes cause a rare lipid disorder, sitosterolemia (Berge *et al.* 2000, Lee *et al.* 2001, Hubáček *et al.* 2001a, Heimer *et al.* 2002, Štefková *et al.* 2004). Sitosterolemia is characterized by high intestinal absorption of all sterols (cholesterol, plant sterols and shellfish sterols), and diminished secretion of sterols into the bile. Sitosterolemia patients have high plasma levels of cholesterol and plant sterols and develop xanthomas and premature coronary atherosclerosis (Bhattacharyya and Conner 1974, Bjorkhem *et al.* 1996).

Because of the important role of the ABCG5 and ABCG8 transporters in the absorption and excretion of sterols, the role of these genes in the genetic determination of plasma lipid levels is currently under intensive investigation. Simultaneously, through the screening of the *ABCG5* and *ABCG8* genes for mutations in sitosterolemia patients, polymorphisms in both genes have been reported (Hubáček *et al.* 2001a, Lu *et al.* 2001).

Recently, associations between the Asp19His and Thr400Lys polymorphisms and concentrations of plasma plant sterols (sitosterol and campesterol) have been described (Berge *et al.* 2002). Another two polymorphisms, Ala632Val (Berge *et al.* 2002) and Gln604Glu (Weggemans *et al.* 2002), have been suggested to have an effect on plasma cholesterol levels.

To evaluate the role of the ABCG5 and ABCG8 variants in the genetic determination of plasma lipids, we analyzed non-synonymous polymorphisms in the *ABCG5* (C1810G = Gln604Glu) and *ABCG8* (G55C = Asp19His, A161G = Tyr54Cys, C1199A = Thr400Lys and C1895T = Ala632Val) genes, and searched for associations between the polymorphisms and plasma lipid levels, and between the polymorphisms and plasma lipid changes over a 8 years´ follow-up.

Methods

Subjects

According to the MONICA observations (Škodová, personal communication), the animal fat consumption and subsequently plasma cholesterol levels in the Czech republic decreased by about 10 % between 1988 and 1997. A total of 131 unrelated men and 154 women (respondence rate of 72 %) included in this study represented a 8-year cohort of a 1 % representative Czech population sample aged 25-64 years, and selected in 1988 as part of the MONICA study (*Multinational Monitoring of Trends and Determinants in Cardiovascular Diseases: "MONICA Project"*. Manual of operations WHO/MNC 82.2, Nov. 1983). These individuals were re-examined in 1996. Written informed consent was obtained from the study participants and the local ethics committee approved the design of the study. Only individuals with all lipid parameters available in both 1988 and 1996 were included in this study.

Table 1. Primer sequences, restriction enzymes and size of the restriction fragments used for detection of polymorphisms in genes for ABCG5 and ABCG8. Mismatched nucleotides are in bold italics.

Polymorphism	Primer sequence	PCR product	Enzyme	Size	Allele
ABCG8	5'atggccgggaaggcggcagaggagag	83 bp	BamH I	83	C (His)
Asp19His	5'acttcccattgctcactcaccgagg***g***at			56 + 27	G (Asp)
ABCG8	5'agggcctccaggatagattgttctcctc	128 bp	Bgl I	128	A (Tyr)
Tyr54Cys	5'ccttgaacccaggcgtgc***gc***ctacctg			102 + 26	G (Cys)
ABCG8	5'agatgcctggggcggtgcagcag***c***tt	108 bp	Afl II	108	C (Thr)
Thr400Lys	5'ggcttaatgtgatatacaaagacttg***g***g			81 + 27	A (Lys)
ABCG8	5'atgtctgtgtctccagatcctcag***g***g	105 bp	Hae III	105	T (Val)
Ala632Val	5'tacaggaccatgaagccaccgctga***c***gcc			79 + 26	C (Ala)
ABCG5	5'aaccacacctgacactgtcaatcttttcct	117 bp	Xho I	117	G (Glu)
Gln604Glu	5'gggcaggttttctcaatgaattgaattcct***c***			86 + 31	C (Gln)

DNA analysis

Three ml of blood collected into EDTA tubes for DNA isolation were diluted with sterile water at a 1:1 ratio and stored at –20 °C. DNA was isolated by a standard method (Miller *et al.* 1988).

The oligonucleotides for amplification of the regions of interests in the *ABCG5* and A*BCG8* genes are listed in Table 1. The PCR mixture (25 µl of total volume) comprised 50 mmol/l KCl, 10 mmol/l Tris (pH 8.3), 1.75 mmol/l $MgCl_2$, 0.2 mmol/l of each dNTP, 0.1 U Taq DNA polymerase, 50 pmol/l of each of primers and ~100 ng of genomic DNA. DNA was amplified on a DYAD (Peltier) PCR device under the following conditions: initial denaturation at 96 °C for 3 min; 35 cycles of 95 °C for 15 s, 65 °C for 30 s, and 72 °C for 30 s, and the final step at 72 °C for 3 min. 15 µl of the PCR product was digested at 37 °C overnight with 5 U of the appropriate restriction enzyme with the appropriate buffer in a total volume of 20 µl (Table 1). The digested PCR products were analyzed in 10 % polyacrylamide microtiter array diagonal gel electrophoresis (Day and Humphries 1994). In 13 individuals, the Thr400Lys polymorphism at the ABCG8 locus was unsuccessfully genotyped even when repeated 3 times.

Lipid analysis

The lipoprotein parameters were measured enzymatically by the WHO Regional Lipid Reference Centre (IKEM, Prague) on a Roche COBAS MIRA autoanalyzer using conventional enzymatic methods with reagents from Boehringer Mannheim Diagnostics and Hoffmann-La Roche, as described in detail elsewhere (Škodová *et al.* 1993). LD-C was calculated by the Friedewald formula (Friedewald *et al.* 1972).

Statistical analysis

Statistical analysis was performed using ANOVA. Triglycerides were logarithmically transformed before the analysis to obtain normal data distribution. If there were fewer than 5 homozygotes for one allele, these were pooled together with the heterozygotes.

Results

Characteristics of the subjects

The basic characteristics of the population studied are summarized in Table 2. There were significant differences between the 1988 and 1996 levels of total and LDL-cholesterol in whole population as well as in males and females, if analyzed separately (see Hubáček *et al*. 2001b,c, 2003 for more details).

Table 2. Basic characteristic of 131 males and 154 females in 1988 and 1996.

	Females 1988	**Females 1996**	**p**
Age (years)	55.5±11.2		
BMI (kg/m^2)	28.2±5.3	29.1±4.1	n.s.
Total cholesterol	5.9±1.1	5.6±1.2	0.01
LDL-cholesterol	3.7±1.0	3.3±1.0	0.01
HDL-cholesterol	1.5±0.3	1.4±0.4	n.s.
Triglycerides	1.5±0.8	1.9± .2	0.01
	Males 1988	**Males 1996**	**p**
Age (years)	55.4±11.6		
BMI (kg/m^2)	27.7±3.8	28.4±4.1	n.s.
Total cholesterol	6.2±1.3	5.4±1.1	0.001
LDL-cholesterol	3.8±1.1	3.3±0.9	0.01
HDL-cholesterol	1.3±0.3	1.2±0.3	n.s.
Triglycerides	2.4±3.4	2.2±1.8	n.s.

Values are given as means ± S.D., cholesterols and triglycerides are in mmol/l, n.s. non-significant

Genotype frequency of the ABCG5 and ABCG8 polymorphisms

Distributions of the *ABCG5* and *ABCG8* genotypes and alleles are summarized in Table 3. The frequencies of the genotypes were in Hardy-Weinberg equilibrium and did not differ between males and females (data not shown). The genotype frequencies are similar to those described previously in European-American populations (Hubáček *et al*. 2001a, Lu *et al*. 2001, Berge *et al*. 2002).

ABCG5 polymorphism and lipid parameters

No association was found between the Gln604Glu polymorphism in the *ABCG5* gene and lipid levels either in the general population or in males or females separately (both in 1988 and 1996). In addition, the changes in lipid levels seen between 1988 and 1996 were not associated with this polymorphism.

ABCG8 polymorphisms and lipid parameters

No association was detected between the Asp19His and Ala632Val polymorphisms in the *ABCG8*

gene and lipid levels either in the general population or in males and females, when analyzed separately. Changes in lipid levels between 1988 and 1996 were not associated with any of these two polymorphisms.

Table 3. Allele and genotype frequencies of the common polymorphisms in ABCG5 and ABCG8 transporters in the Czech population.

Polymorphism	**11**	**12**	**22**		**N (%)**
ABCG8	2	34	249	1- His	38 (6.7 %)
Asp19His	(0.7)	(11.9)	(87.4)	2- Asp	532 (93.3 %)
ABCG8	97	130	58	1- Tyr	324 (56.8 %)
Tyr54Cys	(34.0)	(45.6)	(20.4)	2- Cys	246 (43.2 %)
ABCG8	178	85	9	1- Thr	441 (81.1 %)
Thr400Lys	(65.4)	(31.3)	(3.3)	2- Lys	103 (18.9 %)
ABCG8	24	96	165	1- Val	144 (25.3 %)
Ala632Val	(8.4)	(33.7)	(57.9)	2- Ala	426 (74.7 %)
ABCG5	200	77	8	1- Glu	477 (83.7 %)
Gln604Glu	(70.0)	(27.0)	(2.8)	2- Gln	93 (16.3 %)

Results are given as numbers (%). No differences have been observed between males and females (data not shown).

Table 4. Tyr54Cys polymorphism in ABCG8 in females, their plasma levels of total cholesterol (T-C) and LDL-cholesterol (LDL-C) in 1988 and 1996 and the changes in T-C levels between 1988 and 1996.

	Tyr54Tyr		**Tyr54Cys**		**Cys54Cys**	
N	43		68		28	
Years	1988	1996	1988	1996	1988	1996
T-C	6.0±1.0	5.5±1.1	5.8±1.1	5.5±1.1	5.6±1.1	5.7±1.3
LDL-C	3.7±1.0	3.2±1.0	3.6±0.9	3.3±1.0	3.4±1.0	3.4±1.0
*Δ T-C**	–9.1 %		–5.5 %		+1.8 %	
*Δ LDL-C***	–15.4 %		–9.7 %		+1.2 %	

Data are in mmol/l, means ± S.D., * $p<0.04$, ** $p<0.03$.

The Tyr54Cys polymorphism was not associated with lipid levels in either 1988 or 1996

While no significant change in LDL-cholesterol (Δ +0.04 mmol/l, +1.2 %) was found in females homozygous for the Cys54 allele, a marked decrease was observed in Tyr/Tyr homozygotes (Δ –0.57 mmol/l, –15.4 %) with heterozygotes showing an intermediate decrease (Δ –0.35 mmol/l, –9.7 %) ($p<0.03$, Table 4). Similarly, total cholesterol levels were also significantly reduced in Tyr54 carriers (Tyr/Tyr Δ –0.49 mmol/l, Tyr/Cys, Δ –0.29 mmol/l; Cys/Cys, Δ +0.12 mmol/l, $p<0.04$). In males, no such association between this polymorphism and total cholesterol or LDL-cholesterol was detected.

However, the Thr400Lys polymorphism in *ABCG8* was associated with lipid level changes in males only. The reductions in total cholesterol (Δ –0.90 mmol/l, vers Δ –0.30 mmol/l, $p<0.02$) and LDL-cholesterol (Δ –0.62 mmol/l, vers Δ –0.19 mmol/l, $p<0.04$) in males were significantly greater in Thr400 homozygotes compared to the Lys allele carriers (Table 5).

Additionally, Thr400 homozygotes had a higher level of LDL-cholesterol in 1988 compared to the Lys400 carriers (3.9±1.2 vs. 3.5±0.9 mmol/l, $p<0.05$). However, these differences were no longer present 8 years later, after a significant dietary change, when the plasma

cholesterol levels in the population were lower.

Changes in HDL-cholesterol and TG levels were not significantly influenced by evaluated *ABCG8* polymorphisms.

Table 5. Thr400Lys polymorphism in ABCG8 and plasma levels of total cholesterol (T-C) and LDL-cholesterol (LDL-C) in 1988 and 1996 in males changes of T-C between 1988 and 1996.

	Thr400Thr		**Lys400 carriers**	
N	81		39	
Years	1988	1996	1988	1996
T-C	6.4±1.5	5.5±1.1	5.9±1.0	5.6±0.9
LDL-C	3.9±1.2	3.3±0.9	3.5±0.8	3.3±0.8
*Δ T-C**	–15.0 %		–6.6 %	
*Δ LDL-C***	–18.2 %		–6.1 %	

Data are in mmol/l, means ± S.D., * p<0.02, ** p<0.04

Discussion

No data are available about exact changes of dietary habits in the population under study. On the other hand, as we studied a representative population sample, data applicable to the whole Czech population could be used. The dietary composition changed dramatically in the population of the Czech Republic between 1988 and 1996 (personal communication from the Czech Institute of Agriculture Economy). Briefly, a considerable decrease in the consumption of red meat (79.9 kg/pers/year in 1988 vs. 68.0 kg/pers/year in 1996), eggs (340 vs. 276 no/pers/year), and animal fat (16.2 vs. 9.4 kg/pers/year) was observed, while the consumption of vegetables (70.3 vs. 78.0 kg/pers/year), fruits (63.4 vs. 72.1 kg/pers/year), cereals (113.6 vs. 160.8 kg/pers/year) and vegetable oils (12.2 vs. 15.8 kg/pers/year) increased (Poledne and Škodová 2000). Reflecting these dietary changes, the reduction in total cholesterol level in the population was 0.58 mmol/l ($p<0.0001$) (Poledne and Škodová 2000), and 0.78 mmol/l in this cohort ($p<0.001$). The dietary and lipid changes in the whole population are so robust that the impact of these changes should be expected in practically each individual. In addition, we know there have been no significant changes in factors such as exercise habits, vegetarianism, use of lipid-lowering drugs, or functional food.

ABCG5 and ABCG8 belong to the ABC-transporter superfamily and several of these are involved in lipid transport. Since mutations in *ABCG5* and *ABCG8* have been shown to cause sitosterolemia, a rare autosomal recessive lipid disorder characterized by impaired intestinal sterol absorption and elevated plasma levels of plant sterols and cholesterol, we have proposed that non-synonymous polymorphisms in these genes may influence lipid levels in the general population.

In individuals with high plasma lipid levels, the change in dietary fat intake is the first recommendation by physicians. However, a substantial number of patients show almost no response to dietary changes. There is a subset of patients considered to be "high responders" in which dietary changes result in a marked reduction, while others others show negligible reduction in lipid levels ("non-responders") after such dietary changes. Genetic predisposition is an explanation for this varying response. The ability to differentiate between high responders and non-responders might be important in developing the treatment strategy, since non-responders might benefit from an early onset of lipid-lowering drugs.

A number of studies have evaluated the effect of common polymorphisms on the response of plasma lipids to changes in dietary composition. To date, common variants of *APOA-IV, APOB, APOCIII*, *APOE,* lipoprotein lipase, cholesterol ester transferase, cholesterol 7-α hydroxylase and LDL-receptor have been examined (for review see Ye and Kwiterowich 2000, Masson *et al.* 2003). Most of the studies have focused on the APOE gene and, although the results are not unambiguous, individuals carrying the APOE4 allele seem to be more responsive to dietary changes (for review see Humphries *et al.* 1996). Unfortunately, these results cannot be compared with our study because of the different design. So far, all analysis of gene-associated effect of dietary intervention were carried out on low numbers of healthy preselected individuals (additionally, most of them on males) on a strict diet. In contrast, our study represents a unique population sample, where dietary changes are "evolutionary". All the above genes studied are genes involved in the metabolism of lipids, not in intestinal absorption of sterols. The *ABCG8* and *ABCG5* transporters could directly influence cholesterol absorption in the intestine, thus their variants are likely to be important for the genetic determination of cholesterol absorption and, subsequently, plasma levels of cholesterol.

Indeed, after dietary changes and the accompanying decrease in cholesterol levels in the

population studied, the females homozygous for the Cys54 allele in *ABCG8* showed relatively stable cholesterol levels that did not respond to dietary changes. In contrast, the Tyr54 homozygotes showed the highest change in plasma cholesterol between 1988 and 1996. Although part of the women changed menopausal status between 1988 and 1996, this fact did not influence the effect of polymorphism significantly.

In males, a similar pattern was observed for the Thr400Lys polymorphism at the *ABCG8* locus. The "diet-responsive" allele was Thr400. Homozygotes for this allele had higher LDL-cholesterol levels in 1988 compared to the Lys 400 carriers, and 8 years later, after a significant dietary change, Thr 400 male homozygous showed the highest decrease in cholesterol and LDL-cholesterol over time.

A similar gene-nutrition effect was observed in a recent study of Ordovas *et al.* (2002). The effect of hepatic lipase polymorphism C-514T (on HDL-cholesterol) was found in the population dependent on consumed % of energy from animal fat.

Our study involved a representative Czech population sample where the long-term dietary composition changed, probably as a result of the sweeping political and social changes occurring after 1989. In this sample, variations in the *ABCG8* gene loci (Tyr54Cys and Thr400Lys polymorphisms) were found to play a role in gender-specific reduction in plasma lipid levels as a response to reduced dietary animal fat and cholesterol intake.

Our results suggest that the Tyr54Cys and Thr400Lys polymorphisms in ABCG8 might play a role in the genetic determination of plasma lipids in a gender-specific gene-nutrition manner. These results need to be confirmed in a larger population study.

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Reprint requests

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