The combination of *ApoCIII*, hepatic lipase and hormone sensitive lipase gene polymorphisms suggests an association with susceptibility to gestational hypertension

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Abstract Dyslipidemia and insulin resistance contribute to the endothelial cell dysfunction in hypertensive disorders of pregnancy (HDP) and increase the long-term risk of cardiovascular disease (CVD). The genes linking susceptibility to gestational hypertension (GH) and/or preeclampsia (PE) to the long-term risk of CVD are still unknown. We evaluated the potential association between 14 polymorphisms from six genes involved in lipid metabolism and insulin action and the risk of HDP: namely the lipoprotein lipase (*LPL*), hepatic lipase (*LIPC*), hormone sensitive lipase (*LIPE*), cholesteryl ester transfer protein (*CETP*), *ApoCIII* and *ApoE* gene polymorphisms. Overall, 169 women with HDP [proteinuria (PE) and gestational hypertension without proteinuria (GH)] and 169 controls matched for age and year of delivery were genotyped. Homozygosity of the –514T allele of the –514C > T polymorphism (*LIPC* gene) decreased the risk of GH (OR = 0.17, CI₉₅: 0.02–0.76), while there were more –60G carriers of the –60C > G *LIPE* gene polymorphism (OR = 3.51, CI₉₅: 1.02–12.10) among GH cases, but not in PE cases. The common *ApoCIII* two-locus –482CC/3238CC genotype was lower in women with GH compared with controls (OR = 0.53,

CI₉₅: 0.3–0.9). The combined frequency of at-risk genotypes was higher in cases of GH compared with controls [one at-risk genotype: OR = 3.38 (95% CI: 0.48–41.8); two or more at-risk genotypes: OR = 7.14 (95% CI: 1.21–92.3, *P* = 0.01)], suggesting a gene-dose effect. We conclude that the combined effect of *LIPC*, *LIPE* and *ApoCIII* gene polymorphisms may increase the likelihood of GH, but seemingly not of PE.

Keywords Association study · Candidate gene analysis · Cardiovascular disease · DNA polymorphisms · Gestational hypertension · Preeclampsia

Introduction

Hypertensive disorders of pregnancy (HDP) are characterized by high blood pressure (≥ 140 mmHg/ ≥ 90 mmHg) appearing after 20 weeks of gestation. HDP include hypertension with (preeclampsia, PE) or without (gestational hypertension, GH) proteinuria, defined as >300 mg/24 h (2000; Roberts and Cooper 2001; Roberts et al. 2003; Solomon and Seely 2001; Walker 2000). Overall, these disorders complicate 5–10% of pregnancies in industrialized countries (Cooper et al. 1993; Roberts and Cooper 2001; Solomon and Seely 2001; Walker 2000), and they represent a major cause of fetal and maternal morbidity and mortality (Chappell et al. 2002; Roberts et al. 2003).

There is evidence suggesting that HDP are multifactorial (Roberts and Cooper 2001; Talosi et al. 2000; Thornton and Macdonald 1999), but the etiology and mechanisms causing PE and GH are still undefined. Current pathophysiological hypotheses imply inade-

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quate trophoblastic invasion, oxidative stress, endothelial dysfunction, inappropriate maternal immunological response and genetic susceptibility (Dekker and Sibai 1998; Roberts and Cooper 2001; Sibai et al. 2005; Solomon and Seely 2001; Talosi et al. 2000). A number of studies have described a strong familial trend to developing HDP (Arngrimsson et al. 1995; Chesley et al. 1968; Chesley and Cooper 1986; Salonen Ros et al. 2000), and recently a heritability estimate of 0.47 was observed for HDP in a large sample of over 2,000 twin pairs of women, indicating that HDP, both with proteinuria (PE) and without proteinuria (GH), is highly heritable (Salonen Ros et al. 2000). Published data suggest a significant genetic component to HDP, which may include fetal and environmental factors (Broughton Pipkin 2001; Chesley and Cooper 1986; Cooper 1993; GOPEC Consortium 2005; Roberts and Cooper 2001; Salonen Ros et al. 2000; Thornton and Macdonald 1999). Finally, it is still debated whether PE and GH represent different manifestations of the same entity or rather distinct pathogenic disorders (Brown et al. 2001; Fisher et al. 1981; Seely and Solomon 2003; Vatten and Skjaerven 2004).

Retrospective studies suggest that HDP are associated with many risk factors contributing to cardiovascular disease (CVD) (Jonsson et al. 1995; Roberts 2000). An unfavorable lipid profile associated with an increased CVD risk has also been found in women who suffered from HDP (Forest et al. 2005; Pouta et al. 2004; Roberts 2000; Roberts and Cooper 2001). Triglycerides (TG), free fatty acids (FFA) and low-density lipoprotein cholesterol (LDL-C) concentrations, especially small dense LDL-C, are increased in the presence of HDP, whereas the high-density lipoprotein cholesterol (HDL-C) concentration is decreased (Seely and Solomon 2003). HDP (Chappell et al. 2002; Dekker and Sibai 1998; Roberts 2000; Roberts and Cooper 2001; Seely and Solomon 2003; Solomon and Seely 2001), and GH in particular (Caruso et al. 1999; Roberts et al. 1998), have been shown to be associated with insulin resistance, and women who never developed HDP during pregnancy are at lower risk of CVD (Roberts 2000; Williams 2003; Wilson et al. 2003). Thus, HDP may constitute a first sign of increased CVD risk later in life, possibly through a predisposition to develop the metabolic syndrome that encompasses hypertension, lipid profile alterations, insulin resistance and abdominal obesity (Forest et al. 2005; Pouta et al. 2004; Williams 2003).

Several genetic factors with modest effect probably contribute to PE and/or GH susceptibility. Potential susceptibility genes for HDP involved in thrombophilia (*Factor V Leiden*, *methylenetetrahydrofolate reductase*

and *prothrombin* genes) and blood pressure control (*Angiotensinogen* gene) have been studied, leading to conflicting results (Belo et al. 2004; GOPEC Consortium 2005; Hubel et al. 1999; Kim et al. 2001; Levesque et al. 2004; Roberts and Cooper 2001; Sibai et al. 2005; Walker 2000). Considering the association of dyslipidemia with both HDP and CVD, variants of genes associated with lipid homeostasis have been investigated in small-scale studies (Belo et al. 2004; Hubel et al. 1999; Kim et al. 2001; Roberts and Cooper 2001; Walker 2000), but need to be further explored. Indeed, only a few studies have investigated the genes involved in lipid homeostasis, and only preeclamptic women were included as cases. Hubel et al. 1999 found an association between the N291S polymorphism of the *LPL* gene and the risk of PE in American women, but Kim et al. 2001 were not able to confirm such results in Korean women. Makkonen et al. 2001 did not observe any association between *ApoE* genotypes and the risk of PE in a study of 133 cases and 91 controls. Also, Belo et al. 2004 did not find any associations between *ApoE* and *CETP* polymorphism frequencies between 144 controls and 51 preeclamptic women.

We hypothesized that polymorphisms of genes involved in lipid homeostasis and associated with the action of insulin represent good candidates for bridging GH and/or PE to long-term CVD. In the present study, we aimed to compare the prevalence of 14 polymorphisms from six genes, both individually and jointly, previously associated with lipid metabolism and insulin action, i.e., lipoprotein lipase (*LPL*; D9N, N291S, G188E, P207L, D250N and S447X polymorphisms), hepatic lipase (*LIPC*; -514C > T), hormone-sensitive lipase (*LIPE*, -60C > G), apolipoprotein CIII (*ApoCIII*; -482C > T, 3238C > G) and E (*ApoE*; R112C, C158R) and cholesteryl ester transfer protein (*CETP*; *Taq1B*, R451Q) genes in French-Canadian women with or without HDP.

Materials and methods

Study subjects

The study sample consisted of 169 cases (106 with GH and 63 with PE) and 169 controls matched for maternal age (± 1 year) and year of delivery (± 1 year). They were recruited from a cohort of women studied during pregnancy to identify biological and echographic markers of HDP in nulliparous women (Irion et al. 1998; Masse et al. 1993, 1998). Most subjects were Caucasians (98%, by self assessment of origin) from the Quebec City metropolitan area where 93% of the

population is of French ancestry (Santé Québec 1995). Subjects were classified according to the NHBPEP (National High Blood Pressure Education Program working group) criteria. Briefly, the diagnosis of HDP was defined as a blood pressure of 140/90 mmHg or above on two occasions 6 h apart after 20 weeks of pregnancy, but before labor in women with proven normal blood pressure in the first trimester. Cases of HDP included GH (without proteinuria) and PE [with proteinuria >300 mg/24 h or a positive qualitative results (“dipstick 2+”)]. Blood pressure had to return to normal values ($\leq 120/80$ mmHg) in the post-partum period to be included as cases. Controls had a history of uncomplicated pregnancy. Multiparous women and those with a history of diabetes, renal disease, CVD and those who developed gestational diabetes were excluded. The study was approved by the Hospital’s Ethic’s Review Board, and all subjects gave informed consent.

Analysis of polymorphisms

DNA was extracted from whole blood using the standard protocol of the QIAamp 96 Blood Kit (QIAGEN). The DNA was redissolved in Tris-EDTA buffer (10 mM Tris; 1 mM EDTA; pH 8) and stored at -20°C until genotyping was performed.

All single nucleotide polymorphisms (SNP) were genotyped by an allele-specific oligonucleotide-polymerase chain reaction (ASO-PCR) method optimized in our laboratory. Oligonucleotide pairs were designed for PCR amplification of each SNP studied with the use of the Oligo 4S software (Table 1). The reactions were carried out in a total volume of 25 μl containing 200 μM dNTPs, 0.5 μM of common primer, 0.5 μM of allele-specific primer, 0.4 U *Taq* DNA polymerase (QIAGEN), 1X QIAGEN PCR Buffer, 1X Q-Solution and 20–40 ng DNA. Negative controls without DNA or *Taq* DNA polymerase, as well as positive controls for each genotype, were run with each set of amplification. In general, the initial denaturation was performed at 95°C for 5 min, followed by 32–35 cycles of denaturation at 95°C for 45 s, primer annealing at an optimized temperature (Table 1) for 45 s, extension at 72°C for 45 s and a final extension at 72°C for 7 min. Amplified fragments of each allele were separated on a 1% agarose gel electrophoresis to determine the genotype. For each polymorphism, genotypes were validated by PCR-endonuclease digestion for a subset of samples, and the concordance rate was $\geq 97\%$ for each polymorphism. Three independent readers interpreted each polymorphism. Genotypes without a 100% concordance (3/3) were rejected unless a repeat ASO-

PCR reaction resulted in a 100% concordance. Overall, the concordance rate was $\geq 96\%$ for each polymorphism studied.

Statistics

General clinical characteristics between cases and controls were compared using Student’s *t* tests, χ^2 tests or Fisher tests where appropriate. Genotype frequencies were tested against Hardy–Weinberg equilibrium (HWE) by χ^2 contingency table analysis in the whole study sample, as well as in cases and controls separately. For the two polymorphisms of both *ApoCIII* and *CETP* genes, we estimated the coefficient of linkage disequilibrium D' (D/D_{max} if $D > 0$) $\pm$ SD and *P* value for a two-marker combination using the 2LD program (Zhao et al. 2000). Since allelic frequencies of *LPL* mutations resulting in a severe decrease in *LPL* activity were very low, we combined N291S, D9N, P207L, G188E and D250N polymorphisms to increase the power to detect an association (Ishimura-Oka et al. 1992; Zhang et al. 1998).

Genotype frequencies were compared among all cases together (PE + GH) and separately for GH and PE subgroups and their controls by contingency tables or by the Fisher’s exact probability test where applicable. We performed conditional logistic regression to compute odds ratios (OR) and 95% confidence intervals (CI) in order to test for differences in the prevalence of at-risk genotypes between cases and controls. The frequency of homozygotes for the common allele was considered as the reference for comparisons (OR = 1). Since the body mass index (BMI) is associated with HDP and some of the genotypes under study, all results were adjusted for the pre-pregnancy BMI. Finally, an OR was computed to evaluate the possibility of a joint multi-locus association with HDP, GH and PE of polymorphisms individually associated with HDP ($P < 0.05$) by testing the impact of increasing prevalence of these at-risk genotypes (0, 1, 2 and more). According to the results of individual loci, a recessive or dominant model of gene effect was applied. To test the robustness and consistency of statistically significant results, we performed 2,000 permutation-based simulations of genotypes (Efron and Tibshirani 1995). By permuting the genotype labels, we hypothesize that the association does not depend on the genotype. Thus, we obtained 2,000 new data sets with the genotype (carrier status or combined genotype when applicable) randomly rearranged. We referred to the permutation *P* value as the proportion of permutations out of 2,000 with a *P* value smaller than or equal to the observed *P* value. Since the

Table 1 ASO-PCR primers and annealing temperature for each polymorphism studied

Polymorphisms	ASO-PCR primers (5' → 3')	Annealing temperature
<i>LPL</i> N291S	R-GTTATAGTTTGTGACCCGAG F-CTGGGCTATGAGATCA $\overline{\mathbf{A}}$ (A allele) F-CTGGGCTATGAGATCA $\overline{\mathbf{G}}$ (G allele)	56.0°C
<i>LPL</i> D9N	F-AGAAGACAGAATTAAC $\overline{\mathbf{T}}$ AAGG R-AATTTACTTTTCGATGT $\overline{\mathbf{C}}$ (G allele) R-AATTTACTTTTCGATGT $\overline{\mathbf{T}}$ (A allele)	48.1°C
<i>LPL</i> P207L	R-AGTAACATCTAAAAT $\overline{\mathbf{C}}$ TTTCC F-CATGTTGACATTTACCC (C allele) F-CATGTTGACATTTACCT $\overline{\mathbf{T}}$ (T allele)	51.5°C
<i>LPL</i> G188E	R-GAGGTCAGTGGAGTCTTCCTG F-CACACATTCACCAGAG $\overline{\mathbf{G}}$ (G allele) F-CACACATTCACCAGAG $\overline{\mathbf{A}}$ (A allele)	52.5°C
<i>LPL</i> D250N	R-CTCTGTGCTGATAGCCTAGCC F-TCCATTCATCTCTTCATC $\overline{\mathbf{G}}$ (G allele) F-TCCATTCATCTCTTCATC $\overline{\mathbf{A}}$ (A allele)	50.0°C
<i>LPL</i> S447X	F-GCAGATGCTAAGAGATGGCAG R-GAATGCTCACCAGCCT $\overline{\mathbf{G}}$ (C allele) R-GAATGCTCACCAGCCT $\overline{\mathbf{C}}$ (G allele)	57.5°C
<i>LIPC</i> -514C > T	F-CTCCAGAAGGTAAGAA $\overline{\mathbf{C}}$ CTCC R-AAAACCCCTTACCCCC $\overline{\mathbf{G}}$ (C allele) R-AAAACCCCTTACCCCC $\overline{\mathbf{A}}$ (T allele)	58.0°C
<i>LIPE</i> -60C > G	F-CCTTAATCGGCTTTTGT $\overline{\mathbf{C}}$ CTTCC R-GCCTGCCCAGGGTGTA $\overline{\mathbf{G}}$ (C allele) R-GCCTGCCCAGGGTGTA $\overline{\mathbf{C}}$ (G allele)	55.0°C
<i>ApoCIII</i> 3238C > G (<i>SstI</i>)	R-ACTCAAGTCATGTTGTG $\overline{\mathbf{C}}$ CAGC F-TGCCTATCCATCCTG $\overline{\mathbf{C}}$ C (C allele) F-TGCCTATCCATCCTG $\overline{\mathbf{G}}$ (G allele)	57.7°C
<i>ApoCIII</i> -482C > T	R-GCTGACCAGTGGAGATGAGGG F-CGGAGCCACTGATGCC $\overline{\mathbf{C}}$ (C allele) F-CGGAGCCACTGATGCC $\overline{\mathbf{T}}$ (T allele)	62.0°C
<i>ApoE</i> R158C	R-GGCTCGAACCAGCTCTTGAGG F-CGATGACCTGCAGAAG $\overline{\mathbf{C}}$ (C allele) F-CCGATGACCTGCAGAAG $\overline{\mathbf{T}}$ (T allele)	59.3°C
<i>ApoE</i> C112R	F-CTCATCCCCATCTCGCCCC $\overline{\mathbf{C}}$ CCC R-TACTGCACCAGGCGGCCG $\overline{\mathbf{C}}$ A (T allele) R-TACTGCACCAGGCGGCCG $\overline{\mathbf{G}}$ (C allele)	69.7°C
<i>CETP</i> R451Q	R-TTGGAGACTTCTAGCTCAA $\overline{\mathbf{G}}$ C F-CCTGAGATTATCACT $\overline{\mathbf{C}}$ A (A allele) F-CCTGAGATTATCACT $\overline{\mathbf{G}}$ (G allele)	47.5°C 50.5°C
<i>CETP</i> <i>Taq1B</i>	F-ACACCACTGCCTGATA $\overline{\mathbf{A}}$ CCAT R-GATCTGAACCCTAACT $\overline{\mathbf{C}}$ (G allele) R-GATCTGAACCCTAACT $\overline{\mathbf{T}}$ (A allele)	50.2°C

LPL lipoprotein lipase gene;
LIPC hepatic lipase gene;
LIPE hormone-sensitive lipase gene; *ApoCIII* apolipoprotein CIII gene; *ApoE* apolipoprotein E gene; *CETP* cholesteryl ester transfer protein gene

present study was exploratory, when the permutation *P* value was smaller than 0.05, the null hypothesis of no association was rejected, with the proviso that these results would require validation in subsequent studies. Data were analyzed using SAS, version 8.0 (SAS Institute, Cary, N.C.).

Results

Table 2 summarizes the general characteristics of the study population. Pre-pregnancy BMI, weight gain and systolic and diastolic blood pressures were significantly higher in women who developed HDP compared with matched controls, whereas the gestational age at

delivery was lower. Although within normal range, systolic blood pressure was already higher during the first trimester in women who developed HDP. A family history of hypertension was significantly more frequent in these cases. Smoking was more prevalent among controls.

Hardy–Weinberg equilibrium

In the whole study sample, all (one-locus) genotype frequencies were in Hardy–Weinberg equilibrium (HWE). When χ^2 contingency table analysis was carried out only in women with GH, the -482C > T polymorphism of the *ApoCIII* gene did not follow HWE (*P* = 0.01). Also, in the whole study group, the ob-

Table 2 General characteristics of the study population

Variables	Controls (n = 169)	HDP (n = 169)	Controls (n = 106)	GH (n = 106)	Controls (n = 63)	PE (n = 63)
Age at delivery (years)	27 ± 4 ^a	27 ± 4	27 ± 4	27 ± 4	27 ± 4	27 ± 4
Pre-pregnancy BMI (kg/m ²)	21.9 ± 3.3	24.1 ± 4.3*	21.7 ± 3.0	24.6 ± 4.4*	22.1 ± 3.7	23.3 ± 4.1
Weight gain (kg)	16.3 ± 5.7	19.7 ± 7.7*	16.2 ± 5.7	20.2 ± 8.1*	16.4 ± 5.8	19.0 ± 6.9**
Weeks gestation at delivery	39.4 ± 2.1	38.8 ± 2.3**	39.2 ± 2.3	39.1 ± 2.0	39.6 ± 1.6	38.3 ± 2.7*
Baby's weight (g)	3,335.2 ± 584.9	3,310.6 ± 605.2	3,301.5 ± 611.3	3,423.9 ± 527.9	3,391.4 ± 538.2	3,119.8 ± 679.5**
Blood pressure (mmHg)						
Systolic						
9–12th w	113.0 ± 8.3	119.5 ± 9.8*	112.6 ± 8.8	119.9 ± 10.0*	114.1 ± 7.2	118.5 ± 9.5**
14–18th w	112.6 ± 8.8	119.9 ± 11.1*	112.3 ± 8.0	120.2 ± 11.4*	113.0 ± 10.3	119.2 ± 10.4*
26–28th w	114.9 ± 7.4	122.3 ± 9.1*	115.0 ± 7.0	122.4 ± 9.0*	114.9 ± 8.2	122.1 ± 9.4*
Diastolic						
9–12th w	65.1 ± 7.1	66.9 ± 9.0	64.7 ± 7.4	66.4 ± 9.6	65.8 ± 6.7	67.7 ± 7.8
14–16th w	62.6 ± 7.6	66.6 ± 8.3*	63.0 ± 8.1	66.7 ± 8.8*	61.8 ± 6.7	66.5 ± 7.5*
26–28th w	65.0 ± 7.5	68.6 ± 9.2*	65.6 ± 7.7	68.7 ± 9.1**	63.9 ± 7.1	68.4 ± 9.4**
Family history of PE (%)	4.8	6.2	5 (4.9)	5.8	4.8	6.4
Family history of HDP (%)	29.7	48.2*	31.1	51.5*	27.0	39.7
Smoking ^b (%)	28.4	16.0*	31.1	17.9**	23.8	12.7
Alcohol consumption ^c (%)	3.0	1.2	3.8	1.9	1.6	0

HDP diagnosed as a BP >140/90 mmHg after the 20th week of gestation returning to normal pressure in the postpartum period. There were 106 case control pairs in the gestational hypertension subgroup, and 63 case-control pairs in the preeclampsia subgroup (proteinuria >300 mg/24 h or 2+ dipstick in cases)

BMI body mass index; HDP hypertensive disorders in pregnancy; PE preeclampsia; HBP high blood pressure

* $P < 0.01$; ** $P < 0.05$ (P value based on two-sample Student's t test for continuous variables and χ^2 test for ordinal variables)

^a Values are given as means ± SD

^b More than one cigarette/day during pregnancy

^c More than one consumption/day during pregnancy

served two-locus *ApoCIII* -482C > T and 3238C > G polymorphisms were in LD, resulting in an excess of double heterozygotes (Table 3).

Table 3 Two-locus Hardy–Weinberg and linkage disequilibrium analyses of the combined -482C > T and 3238C > G *ApoCIII* genotype of the study population

Two-locus genotypes: Observed/Expected					
-482C>T	3238C>G				
		CC	CG	GG	
	CC	182/154	2/35	1/2	186/191
	CT	79/97	51/22	1/1	131/120
	TT	6/15	5/4	3/0	14/19
	267/266	58/61	5/3	330 ^a	
D' coefficient of linkage disequilibrium: 0.88516; ^c p value < 0.0001					

ApoCIII apolipoprotein CIII gene; D' coefficient of linkage disequilibrium

^a Observed/expected genotypes according to allele frequencies of the -482C > T and 3238C > G polymorphisms

^b Sum of controls and cases

^c P value determined from 2LD program (Zhao et al. 2000)

LPL, *LIPC* and *LIPE* gene polymorphisms

LPL genotypes

The carrier frequency of the combined *LPL* rare mutations (N291S, D9N, P207L, G188E and D250N) did not differ between controls and women with HDP (9.8 vs. 8.0%, $P > 0.05$) (Table 4). There were no homozygotes and no compound *LPL* heterozygotes in our study sample. The S447X variant, associated with an increased enzymatic activity and a putative protective effect on lipid homeostasis for G allele carriers (Sko-glund-Andersson et al. 2003; van Bockxmeer et al. 2001), was analyzed alone, and there were no differences between controls (allele frequency = 12.1%) and cases (allele frequency = 10.2%) (Table 4).

LIPC genotype

The rare allele frequency of the -514C > T polymorphism of the *LIPC* gene did not differ between controls and cases of HDP (25.9 vs. 21.3%, $P > 0.05$), and the proportion of the three genotypes was not significantly different (Table 4). However, in the GH subgroup, the proportion of -514T homozygotes was

Table 4 Lipoprotein, hepatic and hormone-sensitive lipase gene polymorphisms between cases and controls

Polymorphisms	Whole study sample			GH and controls		
	Controls (n = 169)	Cases (n = 169)	Odds ratio (CI ₉₅) ^a	Controls (n = 106)	Cases (n = 106)	Odds ratio (CI ₉₅) ^a
<i>LPL</i>						
Non-carriers	146	152	1	91	96	1
Carriers ^b	16	13	0.89 (0.39-2.00)	11	9	0.90 (0.31-1.96)
<i>LPL</i> S447X						
CC	133	136	1	83	86	1
CG	31	28	0.84 (0.43-1.66)	20	17	0.72 (0.29-1.78)
GG	5	3	0.46 (0.10-2.08)	3	3	0.82 (0.10-7.12)
<i>LIPC</i> -514C > T						
CC	94	105	1	56	63	1
CT	62	56	0.96 (0.60-1.55)	37	40	1.01 (0.57-1.75)
TT	13	8	0.62 (0.23-1.73)	13	3	0.17 (0.02-0.76)*
<i>LIPE</i> -60C > G						
CC	156	148	1	101	93	1
CG	12	19	1.77 (0.81-3.89)	4	12	3.51 (1.02-12.10)**
GG	0	0	NA	0	0	NA

GH gestational hypertension; *LPL* lipoprotein lipase gene; *LIPC* hepatic lipase gene; *LIPE* hormone-sensitive lipase gene; NA not applicable

* $P < 0.01$; ** $P < 0.05$

^a Conditional logistic regression and 95% confidence intervals adjusted for pre-pregnancy body mass index

^b Combined genotype for N291S, D9N, P207L, G188E and D250N. The frequency of homozygotes for the common allele was considered as the reference for comparisons (OR = 1)

Permutation P value <0.05 shown in bold.

decreased five-fold in cases compared to controls (OR: 0.17; CI₉₅: 0.02–0.76; $P = 0.01$) (Table 4), suggesting a protective effect of the –514TT genotype.

LIPE genotype

The rare allele frequency of the –60C > G polymorphism of the *LIPE* gene did not differ between controls and cases of HDP (3.6 vs. 5.7%, $P > 0.05$), and the proportion of the common –60C homozygotes and –60CG heterozygotes (there were no rare –60GG homozygotes) was not significantly different (Table 4). However, in the GH subgroup, the proportion of heterozygotes was significantly increased in cases compared to controls (OR: 3.51; CI₉₅: 1.02–12.14; $P = 0.03$) (Table 4), suggesting that the rare –60G allele of the *LIPE* gene may be a GH-predisposing gene.

ApoCIII, *ApoE* and *CETP* gene polymorphisms

ApoCIII and *ApoE* genotypes

The rare allele frequencies between controls and cases of HDP were similar for the –482C > T (22.4 vs. 26.5%, $P > 0.05$) and 3238C > G (9.2 vs. 11.5%, $P > 0.05$) variants of the *ApoCIII* gene, and there were

no differences in the proportion of genotypes when both *ApoCIII* polymorphisms were analyzed individually (Table 5). The –482C > T and 3238C > G polymorphisms were in strong linkage disequilibrium ($D' = 0.885$, $P < 0.0001$; Table 3). The common –482CC/3238CC two-locus genotype, the most frequent two-locus genotype of the –482C > T and 3238C > G variants, was more frequent in controls compared with those who developed GH (60.0% vs. 43.8%; $P = 0.01$) (Table 6). The OR for combined non-CC/CC genotypes was 1.88 (CI₉₅: 1.10–3.20) in the GH subgroup, suggesting a protective effect of the frequent two-locus CC/CC genotype. Two-locus comparison between all HDP cases and their controls showed a non-significant trend (OR: 1.51; CI₉₅: 0.99–2.36, $P = 0.054$). Finally, the analysis of *ApoE* variants was negative (Table 5).

CETP genotypes

The rare allele frequencies between controls and cases were similar for the *TaqIB* (42.6 vs. 47.6%, $P > 0.05$) and R451Q (3.6 vs. 2.7%, $P > 0.05$) variants of the *CETP* gene, and there were no differences in the proportion of genotypes when both *CETP* polymorphisms were analyzed individually (Table 5). The *TaqIB* and R451Q polymorphisms were not in linkage

Table 5 Apolipoprotein CIII, apolipoprotein E and cholesteryl ester transfer protein gene polymorphisms between cases and controls

Polymorphisms	Whole study sample			GH and controls		
	Controls (n = 169)	Cases (n = 169)	Odds ratio (CI ₉₅) ^a	Controls (n = 106)	Cases (n = 106)	Odds ratio (CI ₉₅) ^a
<i>ApoCIII</i> -482C > T						
CC	99	86	1	63	50	1
CT	58	75	1.41 (0.90-2.30)	37	53	1.78 (1.03-3.08)*
TT	8	7	0.98 (0.31-3.10)	5	3	0.69 (0.16-2.92)
<i>ApoCIII</i> 3238C > G						
CC	140	129	1	90	81	1
CG	25	34	1.30 (0.70-2.42)	12	21	1.77 (0.85-3.71)
GG	3	2	0.95 (0.14-6.57)	3	1	0.42 (0.04-3.70)
<i>ApoE</i>						
ε3/ε3	86	99	1	52	54	1
ε2/ε2	1	0	NA	1	0	NA
ε2/ε3	31	26	0.77 (0.38-1.56)	19	18	0.97 (0.36-2.58)
ε2/ε4	5	4	0.75 (0.18-3.01)	2	4	2.28 (0.36-14.38)
ε3/ε4	42	34	0.67 (0.37-1.19)	28	25	0.90 (0.43-1.88)
ε4/ε4	2	3	1.94 (0.30-12.42)	2	3	2.26 (0.35-14.55)
<i>CETP</i> TaqIB						
GG	61	47	1	43	30	1
GA	72	82	1.56 (0.91-2.65)	43	53	1.76 (0.90-3.41)
AA	36	39	1.28 (0.69-2.38)	20	23	1.42 (0.62-3.25)
<i>CETP</i> R451Q						
GG	158	160	1	99	102	1
GA	10	9	1.03 (0.39-2.70)	6	4	0.54 (0.14-2.14)
AA	1	0	NA	1	0	NA

GH gestational hypertension; *ApoCIII* apolipoprotein CIII gene; *ApoE* apolipoprotein E gene; *CETP* cholesteryl ester transfer protein gene; NA not applicable

^a Conditional logistic regression and 95% confidence intervals adjusted for pre-pregnancy body mass index. The frequency of homozygotes for the common allele was considered as the reference for comparisons (OR = 1). *Permutation *P* value <0.05 shown in bold

Table 6 Combined two-locus C-482T and C3238G *ApoCIII* genotypes between cases and controls

Genotypes	Whole study sample			GH		
	Controls (n = 169)	Cases (n = 169)	Odds ratio (CI ₉₅) ^a <i>P</i> value ^b	Controls (n = 106)	Cases (n = 106)	Odds ratio (CI ₉₅) ^a <i>P</i> value ^b
<i>ApoCIII</i> (-482C > T/3238C > G)						
CC/CC genotype	99	83	1	63	46	1
Other genotypes	67	85	1.51 (0.99-2.36) <i>P</i> = 0.054	42	59	1.88 (1.10-3.20) <i>P</i> = 0.01

GH gestational hypertension; *ApoCIII* apolipoprotein CIII gene

^a Conditional logistic regression and 95% confidence intervals adjusted for pre-pregnancy body mass index

^b The frequency of double homozygotes for the common allele was considered as the reference for comparisons (OR = 1). Permutation *P* value <0.05 shown in bold

disequilibrium (*D'*: 0.311; *P* = 0.11, data not shown), and two-locus haplotype analysis was non-contributory (data not shown).

Combined frequency of at-risk genotypes

We calculated the combined frequency of at-risk genotypes of polymorphisms that showed a positive association in univariate analyses [assigning the value

“0” to -482CC/3238CC (*ApoCIII*), -514TT (*LIPC*) and -60CC (*LIPE*) genotypes, and the value “1” to other genotypes, i.e., presence of an at-risk genotype] (Fig. 1a). In the whole study sample, the combined frequency of at-risk genotypes was higher in cases compared to controls, but the relationship was not significant. However, in the GH subgroup, the presence of one or two and more at-risk genotypes resulted in ORs of 3.4 (CI₉₅: 0.5–41.8; *P* = 0.28) and 7.1 (CI₉₅:

Fig. 1 Risk of HDP, GH and PE according to the number of at-risk genotypes. **a** Risk of HDP and GH in women with 1 or 2 and more (2+) genotypes compared with those without any at-risk genotype (0) as the reference for comparisons (OR = 1). **b** Risk of PE and GH in women with 2 and more (2+) genotypes compared with those carrying 0 or 1 (0-1) at-risk genotypes as the reference for comparisons (OR = 1). The number of subjects within each at-risk genotype subgroup is indicated below each histogram. Since the number of PE and matched controls was too low in the “0” at-risk genotype category ($n = 2$), OR in this subgroup was calculated only after comparing 0-1 versus 2+ at-risk genotype categories (Fig. 1b). ^aOdds ratios after conditional logistic regression adjusted for body mass index. *Permutation P value = 0.01; **Permutation P value = 0.004 (2,000 permutations were performed). ^b-482CC/3238CC (*ApoCIII*), -514TT (*LIPC*) and -60CC (*LIPE*) genotypes: 0; otherwise 1 (other genotypes, i.e., presence of an at-risk genotype). *HDP* hypertensive disorders of pregnancy; *GH* gestational hypertension; *PE* preeclampsia

1.21–92.3; $P = 0.01$), respectively. Because of the number of PE cases and matched controls in the “0” at-risk genotype category ($n = 2$), it was not possible to test for associations in this subgroup. To allow for more robust comparisons between at-risk genotype categories for both GH and PE subgroups, we tested potential associations between the combined categories “0 or 1” vs. “2 or more (2+)” at-risk genotypes (Fig. 1b). This resulted in about 50% of subjects per at-risk genotype category. In the GH subgroup, although the OR was, as expected, decreased compared with the previous at-risk genotype comparison model, the presence of 2+ at-risk genotypes was significantly associated with the presence of a pregnancy complicated by GH (OR = 2.33; CI₉₅: 1.23–4.42; $P = 0.004$),

while there was no trend in the PE-controls subgroup (OR = 1.06; CI₉₅: 0.51–2.22; $P = 0.89$).

Discussion

The pathogenesis of GH and PE is still undefined, and it is unknown if they represent different manifestations of the same entity or different disorders. There is a growing body of evidence suggesting that exaggerated lipid perturbations and abnormal insulin homeostasis, namely increased blood triglycerides, decreased HDL cholesterol and the presence of atherogenic small, dense LDL-cholesterol more susceptible to oxidation, favor a state of increased oxidative stress contributing to the development of HDP in predisposed women (Hubel et al. 1996; Solomon and Seely 2001; Williams 2003). In this study, we investigated the association between polymorphisms of genes involved in lipid homeostasis and insulin action and HDP, GH and/or PE in a sample of unrelated French-Canadian women. By studying these candidate genes, we were aiming to bridge genetic susceptibility to HDP with the potential long-term risk of CVD in these women.

In the GH subgroup, significant differences were observed. We found a fourfold decrease in the frequency of *LIPC* -514T homozygotes in cases compared with controls (Table 4). Although the role of the hepatic lipase activity and variation of the *LIPC* gene, which bears an insulin-responsive element (IRE) in its promoter region, in CVD risk is controversial and possibly modulated by the metabolic context, environmental factors and genetic background, some data suggest that increased activity of the HL enzyme is related to an atherogenic lipoprotein profile (Deeb et al. 2003). The -514T allele is associated with a 30% reduction of enzymatic activity (Tahvanainen et al. 1998; Zamboni et al. 1998), decreased triglycerides, increased HDL cholesterol, reduced formation of small, dense LDL cholesterol (Chen et al. 2003; Deeb and Peng 2000) and insulin resistance in healthy young males (Jansen et al. 2001). Also, we observed a threefold increased frequency of -60G carriers of the *LIPE* gene (Table 4) in the GH group compared with controls. HSL catalyses the rate-limiting step of adipose tissue lipolysis, and it modulates FFA release by adipocytes (Garenc et al. 2002; Pihlajamäki et al. 2001), insulin action and serum lipid levels (Klannemark et al. 1998; Pihlajamäki et al. 2001). It has been suggested that the -60C > G promoter polymorphism of the *LIPE* gene, which is associated with a 40% variation in promoter activity in vitro (Talmud et al. 1998), may affect the cholesterol level and insulin sensitivity, but the phenotypic effect of this

polymorphism is still unclear and probably depends on metabolic homeostasis, body fat distribution and gender (Pihlajamäki et al. 2001). In the PE-only subgroup, however, we did not find any significant differences compared with controls.

We studied the $-482C > T$ and $3238C > G$ polymorphisms of the *ApoCIII* gene both separately and in combination. The rare alleles of the $-482C > T$ and $3238C > G$ polymorphisms are considered at-risk genotypes because of their association with dyslipidemia. ApoCIII is a non-competitive LPL inhibitor, and *ApoCIII* gene polymorphisms can prevent an insulin effect on *ApoCIII* gene promoter, resulting in inappropriate LPL inhibition and increased plasma TG levels (Talmud and Humphries 1997). In the control group, the $-482C > T$ and $3238C > G$ allele frequencies were similar to what was recently reported in French Canadians, with the $-482C > T$ frequency being lower than in many other Caucasian populations (Garenc et al. 2004). Interestingly, we found more cases of GH carrying the dyslipidemia-associated allele at both loci ($-482T$ allele carriers, OR = 1.7; $3238G$ allele carriers, OR = 1.6), suggesting that women homozygous for both frequent “protecting” $-482CC/3238CC$ *ApoCIII* genotypes were less susceptible to GH. Indeed, the frequency of the protective $-482CC/3238CC$ two-loci genotype was significantly lower in women with GH, resulting in an OR = 1.88 for those bearing the other two-loci genotypes (Table 6). It is noteworthy that by combining the polymorphisms of three independent genes (*LIPC*, *LIPE* and *ApoCIII*) that showed single gene association, we observed that the risk of GH was additive, while there was no trend in the PE subgroup (Fig. 1).

It is also noteworthy that the *ApoCIII* $-482C > T$ and $3238C > G$ polymorphisms were in strong linkage disequilibrium ($D' = 0.89$; $P < 0.0001$), as was recently observed in French Canadians ($D' = 0.80$; $P < 0.0001$) (Garenc et al. 2004). In the present study, the frequency of *ApoCIII* $-482C > T$ and $3238C > G$ double heterozygotes was about 2.5-fold higher than expected (15.5% vs. 6.7%; Table 3). From a study of unrelated adult Caucasians (Dammerman et al. 1993), we determined that the *ApoCIII* $-482C > T$ and $3238C > G$ polymorphisms were also in LD, resulting in a twofold increase of double heterozygotes (11.5% of double heterozygotes observed vs. 5.1% expected). The molecular basis of the observed excess of double heterozygotes for this two-locus *ApoCIII* genotype suggests a selective advantage for double heterozygous carriers and needs to be further explored.

Allelic association studies represent a powerful approach to investigate the genetics of complex traits,

but the possibility of artifactual associations must be taken into account. Other genes currently under investigation are likely to be involved in GH and PE predisposition such as those associated with blood pressure control (Morgan and Ward 1999), thrombophilia (Kupferminc et al. 2000; Ozcan et al. 2001) and oxidative stress (Broughton Pipkin and Roberts 2000; Wang and Walsh 1996), but results are contradictory, possibly due to differences in study design, genetic heterogeneity of the disorder, the genetic background of the populations studied, as well as genetic stratification and type I error (GOPEC Consortium 2005). The French-Canadian population is characterized by a founder effect (Heyer and Tremblay 1995). It is expected to show little admixture and may be less exposed to false-positive results due to genetic stratification. Also, we obtained a very good recruitment rate (85%), limiting the effect of possible bias. Generalization from this prospective study needs to be demonstrated.

Classically, GH is considered as a milder form of the disorder and has been much less extensively studied than PE. Although GH and PE may represent different manifestations of the same disease process, some evidence suggests that GH, mild PE and severe PE may be pathophysiologically distinct entities (Brown et al. 2001; Fisher et al. 1981; Vatten and Skjaerven 2004). Our results suggest that GH and PE could represent different expressions of a spectrum of pathophysiological processes. Interestingly, previous work supported the hypothesis that GH, rather than PE, was associated with insulin resistance (Caruso et al. 1999; Roberts et al. 1998). Furthermore, we (Forest et al. 2005) and others (Pouta et al. 2004) have previously observed that differences in fasting insulin concentrations between women with a past history of HDP and those with normal pregnancy were mostly due to a 10–15% higher insulin concentration in those with prior GH compared with PE. We previously showed, in women in their mid-30s, that 25% of those with a past history of GH had an HDL-C < 1.00 mmol/l and triglycerides ≥ 2.0 mmol/l compared to 14% of those with prior PE (Forest et al. 2005). Genetic variations at the *LIPC*, *LIPE* and *ApoCIII* loci may partly explain the link between GH and CVD risk.

If they are confirmed, our findings strongly suggest that the combined effect of polymorphisms bearing modest individual susceptibility from independent genes involved in lipid metabolism and insulin action, namely *ApoCIII*, *LIPC* and *LIPE* genes, increases the likelihood of GH by 6- to 8-fold, favoring a distinct etiopathogeny between GH and PE. It is thus possible that GH- and PE-associated CVD risks later in life

involve different pathophysiological pathways, but more studies of unrelated subjects with detailed questionnaires focusing on environmental exposure and life habits need to be undertaken to better identify genetic determinants predisposing women to GH and PE, and possibly increasing their risk of CVD later in life.

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