A polygenic basis for four classical Fredrickson hyperlipoproteinemia phenotypes that are characterized by hypertriglyceridemia

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Numerous single nucleotide polymorphisms (SNPs) have been found in recent genome wide association studies (GWAS) to be associated with subtle plasma triglyceride (TG) variation in normolipidemic subjects. However, since these GWAS did not specifically evaluate patients with rare disorders of lipoprotein metabolism—‘hyperlipoproteinemia’ (HLP)—it remains largely unresolved whether any of these SNP determinants of modest physiological changes in TG are necessarily also determinants of most HLP phenotypes. To address this question, we evaluated 28 TG-associated SNPs from GWAS in 386 unrelated adult patients with one of five Fredrickson phenotypes (HLP types 2A, 2B, 3, 4 and 5) and 242 matched normolipidemic controls. We found that several SNPs associated with TG in normolipidemic samples, including APOA5 p.S19W and -1131T>C, TRIB1 rs17321515, TBL2 rs17145738, GCKR rs780094, GALNT2 rs4846914 and ANGPTL3 rs12130333, were significantly associated with HLP types 2B, 3, 4 and 5. The findings indicate that: (i) the TG-associated Fredrickson HLP types 2B, 3, 4 and 5 are polygenic traits; (ii) these Fredrickson HLP types share numerous genetic determinants among themselves; and (iii) genetic determinants of modest TG variation in normolipidemic population samples also underlie—to an apparently even greater degree—susceptibility to these rare HLP phenotypes. Thus, the TG-associated Fredrickson HLP types 2B, 3, 4 and 5, although historically considered to be distinct are actually complex traits sharing among them several common genetic determinants seen in GWAS of normolipidemic population samples.

INTRODUCTION

Genome-wide association studies (GWAS) have generated excitement due to the new possibilities of better understanding the genetic architecture of complex diseases (1–4), such as hyperlipidemia (5). One concern raised about GWAS in general is that the magnitude of genetic associations is relatively modest in scale, and thus possibly of limited interest or importance biologically or medically (1). For the quantitative trait of plasma lipoproteins, many familiar and new loci have emerged from recent GWAS (6–14), however, the magnitude of the genetic effects in normolipidemic samples were very modest—to an extent that many medical practitioners might question their relevance (5). One piece of evidence that would enhance the perception of the potential clinical value of these single nucleotide polymorphisms (SNPs) would be demonstration of their association with disorders that clinicians routinely diagnosis and manage.

The Fredrickson or World Health Organization International Classification of Diseases ontology of primary hyper-
lipoproteinemia (HLP) types have been used as clinical and biochemical ‘shorthand’ by generations of physicians (15). Most Fredrickson types (Supplementary Material, Table S1), specifically HLP types 1, 2B, 3, 4 and 5 (MIM 238600, 144250, 107741, 144600 and 144650, respectively) feature elevated plasma triglyceride (TG) concentration as part of their definition (5,15–17). The exception is familial hypercholesterolemia [FH; HLP type 2A (MIM 143890)], which is often due to heterozygous mutations in LDLR encoding the low-density lipoprotein (LDL) receptor (18). In rare instances, single gene mutations have been found in hyperchylomicronemic patients with HLP type 1, particularly homozygous mutations either in LPL, which encodes lipoprotein lipase (LPL), or in APOC2, which encodes its cofactor apolipoprotein (apo) C-II (19). Thus, the genetic bases of HLP types 3 requires homozygosity for the APOE E2 isoform as a necessary, but not sufficient, genetic condition for expression of the phenotype (5).

We previously found that: (1) APOA5 variants -1131T>C and p.S19W are frequently present in and strongly associated with Fredrickson types 2B, 3, 4 and 5 and also with hypertriglyceridemia (HTG) in general lipid clinic patients (21); and (2) several common SNPs, found to be contributors to subtle variation in TG concentration in normolipidemic controls, including SNPs in APOA5, APOE, TRIB1, TBL2, GCKR and GALNT2 were significant determinants of HLP type 5 (22). On the basis of these results, we hypothesized that common variants recently associated with variation in relatively normal plasma TG levels identified in GWAS (10–15) would also be associated with the remaining uncharacterized Fredrickson HLP phenotypes—namely types 2B, 3 and 4.

RESULTS

Clinical, biochemical and genetic attributes in HLP cases and controls

Baseline demographic attributes of the 368 HLP patients and 242 normolipidemic controls are shown in Table 1. Genotype counts and frequencies among patients and controls are shown in Supplementary Material, Table S2. Minor (i.e. less common) allele frequencies for each genotype in severe HTG cases and controls are shown in Table 2. Frequencies of each genotype did not indicate deviations from Hardy–Weinberg equilibrium. Univariate odds ratios (ORs) for association between SNP genotypes—assuming dominant and recessive models—with the discrete HLP traits are shown in Supplementary Material, Table S3.

Linkage disequilibrium and index SNPs for each locus

Pairwise linkage disequilibrium (LD) was determined across loci (Supplementary Material, Table S4). Of the 28 variants genotyped, 17 index genotypes were selected for further statistical modeling by including only one member of a SNP genotype pair for which LD was significant and the LD correlation coefficient >0.65. Index genotypes that were selected for multivariate regression analysis are indicated in Table 2.

Polygenic determinants of Fredrickson HLP phenotypes: multivariate regression analysis

To assess the relationship of genotypes with HLP phenotypes, dominant and recessive models of minor allele genotypes were tested for each gene using univariate analysis (Supplementary Material, Table S2). For each significant univariate association, either the dominant or recessive variable clearly provided the stronger association; this variable was then used in subsequent analyses for the genotype.

The multivariate ORs for each HLP phenotype individually, all HLP types (2A, 2B, 3, 4 and 5) and all HTG-containing HLP types (2B, 3, 4 and 5) were calculated using the Wald statistic in multivariate logistic regression analysis with stepwise addition of variables, with P < 0.05 set for each step (Table 3A and B). Each of the five models evaluated included index genetic variables only (Table 3A), except for the model for HLP type 3, in which APOE genotype was excluded, since all those subjects had the E2/2 genotype. Age and sex were included in a series of post hoc models (Table 3B).

Significant associations are shown in Table 3A and B. Importantly, none of the genotypes are associated with HLP 2A, a trait that was defined by elevated LDL cholesterol with no elevated TG component. The C-statistic, which corresponds to the area under the receiver–operator characteristic curve for a diagnostic test, was between 0.75 and 0.80 for this combination of index genotypes (Tables 3A and B), APOA5 p.S19W and -1131T>C, TRIB1 rs17321515 and TBL2 rs17145738 were significantly associated with HLP types 2B, 3, 4 and 5. GCKR rs780094 was associated with HLP types 4 and 5 only. GALNT2 rs4846914 was associated with HLP types 4 and 5 only. LPL p.S447X was only associated with HLP type 4, LIPC rs477501 was only associated with HLP type 2B and ANGPTL3 rs12130333 was only associated with HLP type 5. APOE non-E3 genotype was a defining feature of HLP type 3, but was also associated with HLP type 5 in the multivariate model. When all HTG-containing HLP types were considered together, APOA5 p.S19W and -1131T>C, TRIB1 rs17321515, TBL2 rs17145738, GCKR rs780094, GALNT2 rs4846914 and ANGPTL3 rs12130333 were significantly associated in the multivariate logistic model. Inclusion of age and sex resulted in even higher C-statistics: >0.8 in most models tested (Table 3B).

DISCUSSION

The principal novel findings in this study are: (i) several genotypes that were found by GWAS to be associated with moderate variation in plasma TG in samples without severe dyslipidemia are also associated with classical HLP phenotypes; (ii) specifically, four genotypes, namely APOA5 p.S19W and -1131T>C, TRIB1 rs17321515 and TBL2 rs17145738, were significantly associated with HLP 2B, 3, 4 and 5; (iii) other genotypes, including APOE isoforms, GCKR rs780094, GALNT2 rs4846914 and ANGPTL3 rs12130333.
rs12130333 also had significant associations with one or two of these HLP types; and (iv) genotypes contributed to a substantial portion of susceptibility to both the discrete HLP types and to plasma TG concentrations across all HLP types. The findings of SNPs outside of APOA5 (21) associated with HLP types 2B, 3 and 4 are completely novel to the current experiments, although the current results for HLP type 5 essentially confirm our previous findings (22).

Together, the previous (21,22) and current findings emphasize the complex, polygenic nature of Fredrickson HLP types 2B, 3, 4 and 5 (5), and further demonstrate that loci identified in GWAS of normolipidemic samples are also determinants of hyperlipidemia.

The specific SNP genotypes selected for this study were based on GWAS results. In each case, the allele that was associated with the HLP phenotype in our study was also associated in GWAS of normolipidemic samples are also determinants of hyperlipidemia. Asterisk (*) indicates index marker selected for multivariate regression analyses (see text for Materials and Methods).
the GWAS with higher plasma TG concentration. Our study indicates that these common—and so far mechanistically undefined—markers and loci are strongly and cumulatively associated with four different Fredrickson TG-associated HLP phenotypes and four of these genotypes are common across all types evaluated. This further suggests that rare loss-of-function variants in these genes, or in proximal genes for which the SNPs are markers, might also be determinants of severe HTG. Re-sequencing of genes marked by these SNPs should be considered in patients with HLP. But although the findings clearly link these genotypes with dyslipidemia, other factors must also be important, since genotypes studied here cumulatively accounted for only ~20% of variation in plasma TG concentrations.

Our findings are consistent with the emerging model that at the extremes of a complex genetic trait, such as patients with Fredrickson HLP phenotypes are found the cumulative contributions of multiple common alleles. Furthermore, some patients, such as most of those with HLP types 1 and 2A, and ~10% of those with HLP type 5 (20), have rare loss-of-function mutations with large effect sizes. We do not suggest that the variants studied here are directly causative for HLP phenotypes. However, the present study substantially increases the proportion of patients with Fredrickson HLP phenotypes who have a significantly associated underlying genetic predisposition. The findings reinforce that the genetic contribution to most of the Fredrickson HLP phenotypes is complex, but also suggest that additional genes or non-genetic factors may still play an important role. These might be found by performing GWAS in HLP samples themselves, as it remains possible that there might be certain determinants of these abnormal phenotypes that are not necessarily determinants of plasma lipoprotein variation within the normal range. Furthermore, the results show that significant associations can be identified by studying a relatively small number of subjects with extreme values of a quantitative lipoprotein trait. Finally, re-sequencing genes at GWAS loci may reveal new rare loss-of-function mutations.

### MATERIALS AND METHODS

#### Subjects

We studied 386 consecutive unrelated subjects of European ancestry from a tertiary referral lipid clinic. Patients underwent a complete medical history and examination; basic clinical, biochemical and demographic variables were collected. Normolipidemic adult controls were taken from the European subgroup of the Study of Health Assessment and Risk in Ethnic groups, a survey of cardiovascular risk factors in Canadian sub-populations (23) together with healthy population-based controls from Ontario. Using a validated sampling strategy (23), households of Caucasian ethnicity within essentially the same geographic locale as the catchment area from which the patients were referred were randomly selected and mailed an introductory letter, followed by up to 12 telephone calls inviting the individual with the earliest date of birth from the household to participate. All patients provided informed consent for DNA analysis (University of Western Ontario Institutional Review Board protocol #07920E).

| Table 3. Multivariate logistic regression analysis of TG-associated SNPs |
|-----------------------------------------------|----------------|----------------|----------------|----------------|----------------|
|                                              | HLP type 2B    | HLP type 3    | HLP type 4    | HLP type 5    | All HTG types  |
| (A) In Fredrickson HLP types                 |                |               |               |               |                |
| APOA5 p.S19W dominant                        | 4.12 (1.84, 9.23) | 6.47 (2.63, 15.9) | 3.69 (1.35, 10.1) | 7.35 (3.82, 14.2) | 5.35 (2.97, 9.63) |
| APOA5 -1313T>C dominant                      | 2.40 (1.02, 5.61) | 4.56 (1.85, 11.2) | 6.13 (2.62, 14.3) | 5.34 (2.84, 10.0) | 3.99 (2.31, 6.87) |
| TRIB1 rs17321515 G recessive                 | 0.14 (0.05, 0.42) | 0.15 (0.04, 0.68) | 0.23 (0.07, 0.71) | 0.36 (0.18, 0.71) | 0.24 (0.14, 0.43) |
| TBL2 rs17145738 T dominant                  | 0.24 (0.10, 0.59) | 0.36 (0.14, 0.98) | 0.19 (0.06, 0.57) | 0.28 (0.15, 0.55) | 0.30 (0.18, 0.50) |
| GCKR rs780094 A recessive                    | 2.74 (1.41, 5.32) | NS             | NS             | 2.38 (1.31, 4.31) | 2.20 (1.34, 3.62) |
| LIPC rs477501 C dominant                     | 2.04 (1.14, 3.66) | NS             | NS             | NS             | NS             |
| GALNT2 rs4846914 G recessive                 | NS             | NS             | 3.29 (1.36, 7.96) | 2.98 (1.53, 5.82) | 2.43 (1.34, 4.33) |
| LPL S447X dominant                           | NS             | NS             | 0.17 (0.04, 0.86) | NS             | NS             |
| ANGPTL3 rs12130333 T dominant                | NS             | NS             | 0.56 (0.33, 0.94) | 0.61 (0.41, 0.92) | NS             |
| APOE non-E3                                  | NS             | Not tested     | NS             | NS             | 1.99 (1.38, 2.96) |
| SF4 rs10401969 C dominant                    | NS             | NS             | NS             | NS             | NS             |
| C-statistic                                  | 0.767          | 0.749          | 0.778          | 0.806          | 0.790          |
| (B) In Fredrickson HLP types with age and sex|                |               |               |               |                |
| APOA5 p.S19W dominant                        | 5.15 (2.15, 12.36) | 6.41 (2.49, 16.5) | 4.51 (1.42, 14.3) | 7.22 (3.59, 14.5) | 5.23 (2.81, 9.75) |
| APOA5 -1313T>C dominant                      | 3.30 (1.26, 8.63) | 5.62 (2.11, 14.9) | 11.7 (4.23, 32.7) | 6.86 (3.38, 13.9) | 4.98 (2.70, 9.18) |
| TRIB1 rs17321515 G recessive                 | 0.13 (0.04, 0.41) | 0.15 (0.03, 0.66) | 0.19 (0.06, 0.65) | 0.37 (0.19, 0.74) | 0.26 (0.14, 0.44) |
| TBL2 rs17145738 T dominant                  | 0.22 (0.09, 0.57) | 0.28 (0.10, 0.80) | 0.16 (0.05, 0.53) | 0.24 (0.12, 0.49) | 0.25 (0.15, 0.43) |
| GCKR rs780094 A recessive                    | 2.33 (1.16, 4.70) | NS             | NS             | 2.35 (1.26, 4.40) | 2.12 (1.26, 3.58) |
| GALNT2 rs4846914 G recessive                 | 2.34 (1.26, 4.35) | NS             | NS             | NS             | NS             |
| LIPC rs477501 C dominant                     | NS             | NS             | 3.10 (1.49, 6.46) | 2.58 (1.37, 4.82) | NS             |
| ANGPTL3 rs12130333 T dominant                | 0.50 (0.26, 0.96) | NS             | NS             | 0.50 (0.29, 0.86) | 0.55 (0.36, 0.85) |
| APOE non-E3                                  | NS             | Not tested     | NS             | 1.78 (1.17, 2.72) | NS             |
| SF4 rs10401969 C dominant                    | NS             | NS             | NS             | NS             | NS             |
| Male sex                                     | NS             | 2.23 (1.03, 4.87) | 6.38 (2.63, 15.5) | 2.42 (1.43, 4.10) | 2.16 (1.43, 3.28) |
| Age >60 years                                | 2.57 (1.35, 4.90) | NS             | 2.75 (1.25, 6.05) | NS             | NS             |
| C-statistic                                  | 0.805          | 0.778          | 0.855          | 0.833          | 0.813          |

HTG, hypertriglyceridemia; NS, not significant.
Biochemical determinations and classification by HLP phenotype

Plasma lipoprotein profiles were determined as described for adult (age >18 years) lipid clinic patients (21) and for normal controls (23). Subjects were classified as having familial hypercholesterolemia (FH; HLP type 2A) based on the presence of definite diagnostic criteria (24), which in all cases included demonstration of heterozygosity for a disease-causing mutation. Subjects were classified as having HLP type 2B (combined hyperlipidemia) on the basis of both cholesterol and TG higher than age- and sex-specific 95th and 90th percentile values, respectively, together with the presence of cholesterol or TG higher than age- and sex-specific 90th percentile values in a blood relative. Subjects were classified as having HLP type 3 (dysbetalipoproteinemia) based on the presence of an APOE E2/E2 homozygous genotype, TG exceeding age- and sex-specific 90th percentile values and/or documentation of a ratio of very-low density lipoprotein cholesterol to TG ≥0.30, determined as described (21). Subjects were classified as having HLP type 4 (primary HTG) based on TG concentrations exceeding age- and sex-specific 90th percentile values, but not exceeding 10 mmol/l, with no documented chylomicronemia and absence of other lipoprotein phenotypes. Subjects were classified has having HLP type 5 (mixed hyperlipidemia) based on fasting plasma TG >10 mmol/l documented on ≥2 occasions with documented chylomicronemia. We excluded children with fasting plasma TG >10 mmol/l with documented chylomicronemia and homozygous or compound heterozygous mutations in LPL or APOC2.

DNA analysis

DNA was extracted as described (25). Markers were selected based on their association (or the association of their genetic locus) with plasma TG in at least two reports (6–14). APOA5 p.S19W and -1131C>T (dbSNP rs662799), APOC3 482C>T, APOE isotypes (26) and LPL p.S447X (20) were genotyped as described. Genotypes of APOA5 rs6589566 and rs12286037, BUD13 rs964184 and rs28927680, TRIB1 rs17321515 and rs2954029, TBL2 rs17145738 and BAI3 rs2074755, GCKR rs780094 and rs1260326, CILP2 rs16996148, SF4 rs10401969, LPL rs10503669, rs17482753 and rs6993414, ANGPTL3 rs12130333 and rs1748195, GALNT2 rs4846914 and LIPC rs4775041 were determined using TaqMan based protocols and reagents (Applied Biosystems). We also genotyped LDL cholesterol (not TG)-related SNPs PSRC1 rs599839, LDLR rs6511720, APOB rs693 and SELP rs3917820 using TaqMan based protocols and reagents (Applied Biosystems). For APOE, presence or absence of the common E3/3 genotype was evaluated, except in HLP type 3, since APOE E2/2 isotype was considered to be part of the phenotypic definition. A random 5% of genotypes were repeated on a different day; the concordance rate exceeded 99.9% for all markers studied.

Statistical analysis

The two-sample t-test was used to compare the difference between case and control groups for quantitative traits, although Pearson’s chi-square test was used to compare discrete traits with exact P-values obtained whenever cell sizes <5. Deviations of genotype frequency from the Hardy–Weinberg assumption were assessed using a chi-square test. Maximal likelihood LD was estimated as described (27). To assess the relationship of genotypes with HLP phenotypes, dominant and recessive models of minor allele genotypes were tested for each gene. Since we were most interested in the polygenic nature of these traits, we used a multiple logistic regression model with backward elimination to assess the joint effects of SNP genotypes on HLP phenotypes. For a genotype with frequency 0.20, the study sample afforded statistical power (alpha error level = 0.05) to detect 1.4-, 1.6-, 1.8- and 2.0-fold increases in frequency of 59.1, 85.7, 96.9 and 99.9%, respectively. Statistical significance was taken at a two-sided P-value <0.05 for all comparisons. Most analyses were performed using SAS version 9.1 (SAS Institute), whereas exact tests were performed using StatXact8 (Cytel Inc.).

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