A Genetic Polymorphism in GCKR may be Associated with Low High-Density Lipoprotein Cholesterol Phenotype among Filipinos: A Case-control Study

Rody G. Sy, MD,1 Jose B. Nevado, Jr., MD,2 Eddieson M. Gonzales, MD,3 Adrian John P. Bejarin, MD,2,3 Aimee Yvonne Criselle L. Aman, MD,2,3 Elmer Jasper B. Llanes, MD,1 Jose Donato A. Magno, MD,1 Deborah Ignacia D. Ona, MD,1 Felix Eduardo R. Punzalan, MD,1 Paul Ferdinand M. Reganit, MD,2,3 Lourdes Ella G. Santos, MD,1 Richard Henry P. Tiongco II, MD,1 Jaime Alfonso M. Aherrera, MD,1 Lauro L. Abraham IV, MD,1 Charlene F. Agustin, MD1 and Eva Maria C. Cutiongco–de la Paz, MD2,3

1Department of Medicine, Philippine General Hospital, University of the Philippines Manila
2Institute of Human Genetics, National Institutes of Health, University of the Philippines Manila
3Philippine Genome Center, University of the Philippines Diliman, Quezon City

ABSTRACT

Background. Low levels of high-density lipoprotein cholesterol (HDL-c) is a well-recognized risk factor in the development of cardiovascular diseases. Associated gene variants for low HDL-c have already been demonstrated in various populations. Such associations have yet to be established among Filipinos who reportedly have a much higher prevalence of low HDL-c levels compared to other races.

Objective. To determine the association of selected genetic variants and clinical factors with low HDL-c phenotype in Filipinos.

Methods. An age- and sex-matched case-control study was conducted among adult Filipino participants with serum HDL-c concentration less than 35 mg/dL (n=61) and those with HDL-c levels of more than 40 mg/dL (n=116). Genotyping was done using DNA obtained from blood samples. Candidate variants were correlated with the low HDL-c phenotype using chi-squared test and conditional logistic regression analysis.

Results. Twelve single nucleotide polymorphisms (SNPs) were associated with low HDL-c phenotype among Filipinos with univariate regression analysis. The variant rs1260326 of glucokinase regulator (GCKR) (CT genotype: adjusted OR=5.17; p-value=0.007; TT genotype: adjusted OR=6.28; p-value=0.027) remained associated with low HDL-c phenotype, together with hypertension and elevated body mass index, after multiple regression analysis.

Conclusion. The variant rs1260326 near GCKR is associated with low HDL-c phenotype among Filipinos. Its role in the expression of low HDL-c phenotype should be further investigated prior to the development of possible clinical applications.

Keywords: cardiovascular disease, dyslipidemia, GCKR, genetics, single nucleotide polymorphism, rs1260326
INTRODUCTION

High-density lipoprotein cholesterol (HDL-c) is a strong and independent inverse predictor of cardiovascular disease. In the Lipid Research Clinics Coronary Primary Prevention Trial (LRC-CPPT)\(^1\) and the Framingham Heart Study,\(^2\) high HDL-c levels were associated with decreased risk and incidence of coronary heart disease. On the other hand, the Prospective Cardiovascular Münster (PROCAM) study,\(^3\) the Israel Ischemic Heart Disease Study,\(^4\) and Prospective Epidemiological Study of Myocardial Infarction (PRIME),\(^5\) observed increased cardiovascular risk and mortality associated with low HDL-c levels.

Compared to other populations, Filipinos have a higher prevalence of low HDL-c levels. This could potentially explain why diseases of the cardiovascular system remain top causes of mortality in the Philippines, with 12.7% of total deaths due to ischemic heart diseases and 9.8% due to cerebrovascular disease.\(^6\) According to the National Nutrition and Health Survey conducted in 2013,\(^7\) the prevalence of low HDL-c was 71.3%, affecting 7 out of 10 Filipino adults. In comparison, lower prevalence of low HDL-c was noted among Hispanics (64.1%), non-Hispanic Blacks (40.5%), non-Hispanic Whites (46.9%) and Northwestern Chinese (60.9%).\(^5,8\) These differences in low HDL-c prevalence across ethnicities may be explained by variations in genetic profiles. In addition, Filipinos are underrepresented in previous genetic studies. Knowledge of unique Filipino phenotypes for low HDL-c phenotypes may aid in addressing cardiovascular disease in the Philippines.

Several genetic variants have been associated with HDL-c metabolism in some ethnic groups. In a study involving Sorbs, a Slavic minority in Germany, it was shown that a \(THO\) complex 5 (\(THOC5\)) polymorphism affects HDL-c levels through the regulation of genes involved in HDL-c synthesis such as apolipoprotein A1 (\(APOA1\)), scavenger receptor class B member 1 (\(SCARB1\)), and \(ATP\)-binding cassette transporter G8 (\(ABCG8\)).\(^10\) In another study involving Arabs, variants in \(cholesterol ester transfer protein\) (\(CETP\)) and \(LIM and cysteine-rich domains protein 1\) (\(LMCD1\)) were associated with low HDL-c.\(^11\) Among Hispanics and specifically among Mexicans, a variant in \(ATP\)-binding cassette transporter A1 (\(ABCA1\)) was implicated.\(^12,13\)

However, these findings cannot be generalized across populations due to inter-ethnic variability in genetic profiles. A population-specific study is needed to yield relevant observations to explain unique phenotypes. Therefore, this study aimed to investigate the presence of associated genetic variants and assess the contribution of significant clinical factors with low HDL-c phenotype among Filipinos.

MATERIALS AND METHODS

Study design

We conducted an age- and sex-matched case-control study, with low HDL-c cases defined as participants with at least two documented low HDL-c determinations (either a combination of at least one reading of below 35 mg/dL and one reading below 40 mg/dL or two readings below 35 mg/dL). Controls were defined as those with two documented HDL-c readings of more than 40 mg/dL.

Study Size

Sample size was computed using the recessive model to provide the largest sample size estimate, a minimum minor allele frequency of 20% (\(MAF \geq 0.20\)), and an alpha of 0.05, with a case-to-control ratio of 1:2. The computed minimum sample size was set at 62 cases and 124 controls.

Setting and enrollment of participants

Adult participants were recruited from the ambulatory clinics of the Philippine General Hospital as well as various communities and medical facilities within Metro Manila from July 2013 to March 2017. Such recruitment sites were considered by virtue of the volume as well as variety of patients seen.

Eligibility criteria

The following criteria were used to enroll participants: (a) aged 18 years or older, (b) of Filipino descent up to the 3rd degree of consanguinity, and (c) able to provide independent consent. Participants were excluded if they had any of the following conditions at the time of enrollment: decompensated heart failure, decompensated chronic lung disease, decompensated chronic liver disease, end-stage renal disease, active malignancy, secondary hypertension, secondary dyslipidemia, pregnancy, and 3rd degree of consanguinity within enrolled participants.

Two controls were recruited for every case among individuals from the study population who had normal or elevated HDL-c levels. Controls were individually matched to cases by age and sex. Cases and controls were defined according to the criteria mentioned earlier.

Clinical data collection

Demographic and clinical characteristics of the participants were obtained through interview and chart review. Lipid profile levels were measured and recorded.

Customization of genotyping chip

We used a customized GoldenGate Genotyping (GGGT) beadchip (Illumina, Inc., San Diego, CA, USA), designed in 2012 using candidate single nucleotide polymorphisms (SNPs) from both coding and noncoding regions, including intergenic and intronic SNPs, which have shown evidence of association with lipid transport and
metabolism (Table 1). The following databases were used for extensive search of SNPs: PharmGKB (Pharmacogenomics Knowledgebase) database, NHGRI GWAS Catalog (National Human Genome Research Institute Genome-Wide Association Study), PubMED and patent databases (e.g., Patentscope and Espacenet), where risk and protective odds ratios (OR) were provided. The selected SNPs were scored by Illumina Inc. to determine their suitability to discriminate genetic variants, and to estimate their specificity.

DNA extraction and quantification
We extracted DNA using the QiaAmp DNA Minikit (QIAGEN GmbH, Germany), following the spin protocol for blood buffy coat specified in the manufacturer’s instruction manual. DNA was quantified using a spectrometer at 260 nm and stored at -20°C until use. All DNA samples had absorbance ratio (A_{260} / A_{280}) above 1.80; this is a measure of quality of the sample.

Genotyping
Customized genotyping of candidate SNPs was performed using DNA microarray technology following the GoldenGate Genotyping (GGGT) assay protocol specified in the manufacturer’s manual. After microarray processing, the beadchip was imaged on the HiScan System and data from these images were analyzed using Genotyping module v.1.9.4 (GenomeStudio software version 2011.1, Illumina Inc, USA). Variant selection and genotyping methods were similar to methods done in earlier studies.14-15

Data analyses
Quality control
To ensure high quality data, GenomeStudio and PLINK version 2.05.1016 were used to identify and remove participants and markers that may lead to false-positive and false-negative associations. We included Genotype data from participants with call rates >95% after evaluation with GenomeStudio, and with individual missingness <0.05 (missingness test) in PLINK. The following thresholds were used for the genotype data: minor allele frequency of 0.01 (frequency test), genotype missingness of 0.05 (missingness test), and Hardy-Weinberg Equilibrium (HWE) of controls of p<0.001 (HWE test). A test for epistasis was also done to account for interaction between variants.

Determination of genotypic effect
We performed chi-square tests to assess significant differences among variants of interest, namely, allelic and genotypic association tests (additive, dominant, and recessive models). These models were identified based on the distribution of genotypes. Fisher–Irwin exact tests for allelic and genotypic associations were done, setting the cut-off at Bonferroni-corrected $\alpha$, if applicable.

Logistic regression analysis
Upon determination of models, we recoded genotypes according to the significant model for univariate analysis in Stata 13 (College Station, Texas). Univariate logistic regression analysis was done to determine association of SNPs with low HDL-c phenotype, with cut-off p-value set at 0.05. Multiple logistic regression was performed to assess contribution of associated clinical factors. Quality control and statistical analyses were similar to the methods done in studies published earlier.14-15

Ethics statement
The study protocol was approved by the University of the Philippines Manila - Research Ethics Board. All participants were required to provide written informed consent. Approval was sought from administrators or authorities of the areas where participants were enrolled.

Table 1. Clinical characteristics of study participants

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>% Participants with low HDL-c (n = 61)</th>
<th>% Participants with normal HDL-c (n = 116)</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in years, mean (SD)</td>
<td>50 (10)</td>
<td>52 (10)</td>
<td>0.38</td>
</tr>
<tr>
<td>Males, %</td>
<td>86.9</td>
<td>87.9</td>
<td>0.84</td>
</tr>
<tr>
<td>HDL-c in mg/dL, mean (SD)</td>
<td>33.05 (7.00)</td>
<td>53.64 (11.76)</td>
<td>&lt;0.001†</td>
</tr>
<tr>
<td>Comorbidities, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elevated BMI (&gt;25 kg/m²)</td>
<td>71.4</td>
<td>35.7</td>
<td>&lt;0.001†</td>
</tr>
<tr>
<td>Hypertension</td>
<td>86.9</td>
<td>31.0</td>
<td>&lt;0.001†</td>
</tr>
<tr>
<td>Diabetes</td>
<td>42.6</td>
<td>1.7</td>
<td>&lt;0.001†</td>
</tr>
<tr>
<td>Reduced eGFR (&lt;60 mml/min)</td>
<td>4.9</td>
<td>4.3</td>
<td>0.80</td>
</tr>
<tr>
<td>Lifestyle factors, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td>47.5</td>
<td>40.5</td>
<td>0.33</td>
</tr>
<tr>
<td>Alcohol drinking</td>
<td>78.7</td>
<td>69.0</td>
<td>0.13</td>
</tr>
</tbody>
</table>

*The following were used to obtain the p-values (significance set at p < 0.05): Student’s t-test for age and HDL-c, chi-square test for sex, and conditional logistic regression for comorbidities and lifestyle factors. †statistically significant. HDL-c, high-density lipoprotein; BMI, body mass index; eGFR, estimated glomerular filtration rate

GCKR polymorphism in low HDL phenotype among Filipinos
A total of 186 participants were initially recruited for the study, with 62 low HDL-c participants (cases) and 124 normal HDL-c participants (controls). After quality control, 177 participants (61 cases and 116 controls) remained (Figure 1A).

Among the initial list of 100 SNPs known to be associated with HDL-c metabolism and transport, 42 SNPs were excluded after quality control using Hardy-Weinberg exact test, individual missingness test, and minor allele frequency threshold as criteria. Thirty-eight (38) SNPs were excluded after failing to meet statistical significance on genotypic association analysis using Fisher’s test (Bonferroni-adjusted \( \alpha = 0.00086 \)), and 2 were removed after checking for epistasis. Three (3) SNPs did not reach MAF of 0.10. No SNPs reached statistical significance on Bonferroni-adjusted \( \alpha = 0.00086 \).

Figure 1. Overview of data processing and analysis. A total of 182 participants (A) and 58 SNPs (B) were analyzed to determine association of clinical metadata and genetic variants with low HDL.

Abbreviations: mind, individual missingness; SNP, single nucleotide polymorphism; HWE, Hardy-Weinberg equilibrium; geno, genotypic missingness; MAF, minor allele frequency

RESULTS

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Among the initial list of 100 SNPs known to be associated with HDL-c metabolism and transport, 42 SNPs were excluded after quality control using Hardy-Weinberg exact test, individual missingness test, and minor allele frequency threshold as criteria. Thirty-eight (38) SNPs were excluded after failing to meet statistical significance on genotypic association analysis using Fisher’s test (Bonferroni-adjusted \( \alpha = 0.00086 \)), and 2 were removed after checking for epistasis. Three (3) SNPs have MAF >0.10. Fifteen (15) SNPs were included in the univariate regression analysis (Figure 1B).

More participants in the low HDL-c group have elevated BMI (71.4%), hypertension (86.9%) and diabetes (42.6%). Participants with low HDL-c levels are expected to have higher frequency of the said conditions since these comorbidities are known cardiovascular risk factors. The average HDL-c measurement among cases was 33.05 mg/dL, a much lower value than the controls at 53.64 mg/dL (p<0.001). (Table 1)

Univariate analysis was used to determine the association of the remaining fifteen (15) SNPs with low HDL-c levels. Of these, 12 SNPs (7 SNPs with additive, 3 with dominant, and 2 with recessive genotypic effects) showed statistically significant association (p<0.005) while exhibiting crude ORs above 2.5 after crude analysis (Table 2). This implies their clinical significance for potential application in management of cardiovascular diseases once verified in a Filipino cohort.

Among the twelve variants listed in Table 2, rs662799 in APOA5 had the highest crude OR of 27.88 with a p-value of 0.002. Three variants, namely rs673548, rs6754295 and rs676210, are located in the same locus and are commonly associated with APOB.

Multiple regression analysis was conducted to account for the effect of clinical variables on the association of the genetic variants with low HDL-c (Table 3). Variable selection was done to select the variant most statistically associated with the low HDL-c phenotype. The variant rs1260326 retained its association with low HDL-c phenotype when adjusted for the presence of hypertension and elevated BMI (Table 3).

Type 2 diabetes mellitus (T2DM) is also known to be associated with low HDL-c. In this study, we did post-hoc matching for T2DM and attempted to include the variable into the equation. Of the 28 participants with T2DM, 26 were shown to have low HDL-c. T2DM conferred 41 times higher odds of having low HDL-c on exact logistic regression analysis (95% CI 9.58, 376.75; p<0.0001). Adjusting for T2DM on conditional logistic regression analysis showed increased odds ratios for both additive and dominant model (Table 3). However, it was observed that the confidence intervals widened. This may be due to the difference in distribution of the participants with and without T2DM (Table 4), and the conditional regression model used for matched samples.

A post-hoc subgroup analysis was done to observe the association of the SNP with low HDL-c separately among those with T2DM and those without the T2DM (Table 4). The exact logistic regression analysis done to check association among participants with T2DM may have been limited by the small sample size; however, it was observed
that all diabetic participants with the risk allele T had low HDL-c. Among participants without T2DM, both the additive and dominant models of genotypic effect showed increased odds of having low HDL-c among those with the risk allele T. These show that the association of the SNP with low HDL-c may be independent of an individual’s T2DM status.

**DISCUSSION**

Twelve variants located in APOA5/B, N-acetylgalactosaminyl-transferase 2 (GALNT2), ATP binding cassette subfamily G member 2 (ABCG2), dipeptidase 3 (DPEP3), cholesteryl ester transfer protein (CETP), dedicator of cytokinesis protein 1 (DOCK1), and glucokinase regulator (GCKR) are found to

be associated with low HDL-c phenotype in the Filipino cohort in this study. One genetic variant, rs1260326 in GCKR, retained its statistically significant association when adjusted for the presence of hypertension and elevated BMI.

The GCKR gene, located in chromosome 2p, codes for glucokinase regulator protein (GKRP) that is predominantly involved in glucose metabolism in the liver. GKRP modulates the activity of glucokinase, an enzyme that phosphorylates glucose during the first step of glycolysis and glycogen synthesis. GKRP binds glucokinase and relocates it from the cytoplasm to the nucleus where it is held inactive. Failure in GKRP modulation could result to enhanced glycolysis and increased de novo lipogenesis in the liver. Variations in GCKR have been reported to be associated with dyslipidemia and changes in metabolic profile.

A missense variant in the isomerase domain of GCKR, rs1260326, likely results to conformational changes in GKRP affecting binding to glucokinase. Impaired binding may result in uninhibited glycolysis, which may increase lipogenesis in the liver, especially of triglyceride (TG). Studies by Chasman et al., Teslovich et al., and Weissglas-Volkov et al. showed that the variant was significantly associated with high TG levels. A review by Welty mentioned that higher TG levels facilitate more CETP-mediated transfer resulting in a higher concentration of TG-rich HDL-c. Compared to cholesteryl ester-rich TG, TG-rich HDL-c is more rapidly metabolized, leading to lower levels of HDL-c. A previous study by Zhou et al. among Chinese patients has already implicated the variant with low HDL-c levels. However, Varbo and colleagues showed in a cross-sectional study that the SNP is minimally associated with low HDL-c, with note of a stepwise decrease as the number of T alleles increase (~0.01 mmol/L; p = 0.02). Among Filipinos, those who have the TT or TC genotype are likely to have 3.59 times higher odds of having low HDL-c levels than those with the CC genotype.

The risk allele (T allele) frequency of rs1260326 among the study controls was 18%, which is similar to that in South Asians (20%), higher than Africans (9%), and lower than admixed Americans (36%), Europeans (41%), East Asians (48%).

Knowing the role of GCKR in glucose metabolism, the presence of concomitant diabetes mellitus as a possible confounder was tested. Although a larger sample size for subgroup analyses among participants with diabetes and those without diabetes is recommended, the current analysis showed a trend that the association of rs1260326 and low HDL-c persists, regardless of DM status. For subsequent studies, it may be prudent to recommend that T2DM status (with or without T2DM), as well as control of T2DM be included as a priori variables for analysis.

The clinical factors found to be associated with low HDL-c phenotype in this study population are hypertension and elevated BMI. These are typical features of metabolic syndrome, which is a cluster of risk factors for cardiovascular disease. The group of Liu et al. has characterized the link between low HDL-c and other components of metabolic syndrome. They found that both men and women with initially low HDL-c develop hypertension as the most common additional and new-onset metabolic syndrome component. They purported that low HDL-c concentration is connected to other components of the metabolic syndrome, mainly as a consequence of systemic low-grade inflammation and APOA1 dysfunction. The association between hypertension and HDL-c levels is further substantiated in their opposing effects in the number of circulating endothelial progenitor cells in the vascular endothelium. The link between elevated BMI and low HDL-c can be understood in terms of insulin resistance resulting from adipose tissue resistant to the antilipolytic effects of insulin leading to increased hepatic flux of fatty acids from dietary sources and intravascular lipolysis.

This is a hypothesis-generating study and larger follow-up prospective studies are needed to confirm current findings. In addition, the identified SNPs in this study that were not previously associated with low HDL-c in other populations should be interpreted with caution. Generalizing results and applying them to Filipinos will only be recommended upon replication and validation of positive associations among these SNPs.

**CONCLUSION**

The frequency of the variant rs1260326 in GCKR, adjusted for the presence of hypertension and elevated BMI, is significantly associated with low HDL-c phenotype of Filipinos. Further study is recommended to assess the role of the variant in the expression of low HDL-c phenotype among Filipinos. This will provide insight on the prevention and clinical management of cardiovascular diseases.

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**Statement of Authorship**

For conceptualization and methodology: RG Sy, EC Cutiongo-de la Paz, JB Nevado, FR Punaalan, EB Llanes, JA Magno, DD Ona, PM Reganit, LG Santos, and RP Tiongco.

For funding acquisition, project administration and supervision: RG Sy, EC Cutiongo-de la Paz, JB Nevado, and AL Aman.
For investigation: RG Sy, EC Cutiongco-de la Paz, JB Nevada, FR Punzalan, EB Llanes, JA Magno, DD Ona, PM Reganit, LG Santos, RP Tiongco, JM Aherrera, LL Abrahan, CF Agustin, AL Aman, and AP Bejarin.

For data curation and formal analysis: JB Nevada, AL Aman, and AP Bejarin.

Interpretation of data: RG Sy, EC Cutiongco-de la Paz, JB Nevada, FR Punzalan, EB Llanes, JA Magno, DD Ona, PM Reganit, LG Santos, RP Tiongco, JM Aherrera, LL Abrahan, CF Agustin, AL Aman, and AP Bejarin.

Writing of original draft and visualization: JB Nevada, AL Aman, AP Bejarin, and EM Gonzales.

Revising the manuscript for significant intellectual content: RG Sy, EC Cutiongco-de la Paz, and JB Nevada.

All authors approved the final version of the manuscript to be published.

Author Disclosure

The following authors have no conflicts of interest to declare: Dr. Abraham, Dr. Agustin, Dr. Aman, Dr. Bejarin, Dr. Cutiongco-de la Paz, Dr. Magno, Dr. Nevada, Dr. Punzalan, and Dr. Reganit.

Dr. Aherrera: Received honoraria for lectures for Servier, Astrazeneca, GX International.

Dr. Llanes: Received honoraria for lectures for Servier, MSD, Bayer, Astrazeneca, Boehringer Ingelheim, Menarini, Novartis and LRI-Therapharma.

Dr. Ona: Received speaker grants from Astrazeneca, Servier, Viatris, Sanofi and Zydus; Advisory Board Member of Sanofi.

Dr. Santos: Past speaker for the following: Servier, Astrazeneca, MSD, LRI-Therapharma, OEP, Zuellig, Boehringer Ingelheim, Pascual Labs, Kanarib, Menarini, Ajanta, Torrent; Advisory Board Member of Zuellig and Kanarib.

Dr. Sy: Received research grants from MSD, Novartis, Pfizer and Servier; received honoraria for lectures for Astrazeneca, Corbridge, MSD, Novartis, Servier, LRI-Therapharma and Zuellig-Amgen; Advisory Board Member of Astrazeneca, MSD, Novartis, Pfizer, Servier and Zuellig-Amgen.

Dr. Tiongco: Past speaker for CME for ADP Pharma for levamlodipine molecule and 2019 CME module for levamlodipine besylate with ADP Pharma. Past resource speaker on clopidogrel for Sanofi, and made a module for clopidogrel for Getz. Prepared module for best/-limited use for inclusion in clinical practice guidelines.

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