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A polymorphism in *HLA-G* modifies statin benefit in asthma

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Abstract

Several reports have shown that statin treatment benefits patients with asthma, however inconsistent effects have been observed. The mir-152 family (148a, 148b and 152) has been implicated in asthma. These microRNAs suppress *HLA-G* expression, and rs1063320, a common SNP in the *HLA-G* 3'UTR which is associated with asthma risk, modulates miRNA binding. We report that statins up-regulate mir-148b and 152, and affect *HLA-G* expression in an rs1063320 dependent fashion. In addition, we found that individuals who carried the G minor allele of rs1063320 had reduced asthma related exacerbations (emergency department visits, hospitalizations or oral steroid use) compared to non-carriers ($p=0.03$) in statin users ascertained in the Personalized Medicine Research Project at the Marshfield Clinic ($n=421$). These findings

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

support the hypothesis that rs1063320 modifies the effect of statin benefit in asthma, and thus may contribute to variation in statin efficacy for the management of this disease.

Keywords

pharmacogenetics; HLA-G; statin; asthma; mir-148; mir-152

INTRODUCTION

Statins, or HMG-CoA reductase inhibitors, which are primarily prescribed for the reduction of cardiovascular disease, have well-documented anti-inflammatory effects attributed to the inhibition of both isoprenoid production and prenylation of inflammatory signaling molecules [1]. Thus, statins may be of benefit in the prevention and treatment of asthma, a disease in which inflammation is of central importance [2–5]. Statin exposure has been associated with a reduction in asthma-related hospitalizations, attenuation in the decline of lung function in the elderly and in smokers, and a reduction in the number of Chronic Obstructive Pulmonary Disease (COPD) exacerbations [6–13]. For example, a retrospective cohort study of over 12 million members within the Medco Health Solutions National Integrated database found that adult with asthma and concomitant statin and inhaled corticosteroid therapy had a 33% lower risk of emergency department (ED) visits for asthma compared to non-statin users after adjustment for potential confounding variables [14]. However, two small trials failed to demonstrate improvement in indices of asthma status with statin treatment [15, 16], leading some to question the potential benefit of statins for asthma management [17]. This inconsistency may have a genetic basis as the considerable variability in both the lipid lowering and anti-inflammatory effects of statins is partly due to genetic factors [18, 19].

HLA-G, a non-classic class I HLA molecule with important immunomodulatory properties, has been associated with asthma in multiple independent populations [20–23]. Although the precise mechanism underlying this relationship is unknown, both HLA-G transcript and protein levels have been reported to be increased in the lung and plasma of subjects with asthma [24–26]. In addition, a common SNP (rs1063320 aka *HLA-G* +3142, 0.46 minor allele frequency,) within the 3' UTR of the *HLA-G* gene has been associated with reduced risk of asthma in the children of mothers with asthma [27]. Notably, rs1063320 disrupts the targeting of the mir-152 family (mir-148a, mir-148b and mir-152) to the *HLA-G* gene such that these microRNAs can only down-regulate *HLA-G* transcripts carrying the variant G allele, with no effect on transcripts with the C allele. Furthermore, adult asthmatic patients with an asthmatic mother were also shown to have elevated mir-148b levels in airway epithelial cells, and greater soluble HLA-G (sHLA-G) in bronchoalveolar lavage fluid in GG homozygotes compared to non-carriers [28]. Thus these results support the hypothesis that allele-specific targeting of mir-148a, mir-148b, and/or mir-152 may contribute to the association of the *HLA-G* gene with the risk of asthma.

In this study, we report that statin treatment up-regulates expression of mir-148a, mir-148b and mir-152 *in vitro*, leading to the hypothesis that statin treatment may benefit asthmatic

patients that carry the *HLA-G* G allele by causing a reduction of *HLA-G* expression in these subjects. Thus, we sought to test if rs1063320 modifies statin effects on asthma-related exacerbations in a population-based cohort of statin users identified from the Marshfield Clinic Personalized Medicine Research Program (PMRP).

MATERIALS, SUBJECTS AND METHODS

Cell culture

Immortalized lymphoblastoid cell lines (LCLs) established from participants of the Cholesterol and Pharmacogenetics (CAP) clinical trial were grown under standard conditions in RPMI supplemented with 10% FBS and either 2.0 μ M activated simvastatin or sham buffer for 24 hours as previously described[29]. 5 \times 10⁶ HepG2 cells were exposed in replicate to DMEM supplemented with 10% FBS and either 2.0 μ M activated simvastatin or sham buffer for 24 hours. Simvastatin was obtained as a gift from Merck, and activated as previously described [29].

Gene expression measurements

Total RNA was extracted using the mirVana RNA isolation kit (Ambion, Austin, TX), quantified by NanoDrop (Thermo Scientific, Waltham, MA), and RNA integrity was assessed on the Agilent Technologies 2100 Bioanalyzer. *HLA-G* was quantified by expression array (GEO accession number: GSE36868) as previously described [30]. mir-148a, mir-148b and mir-152 were quantified by real-time PCR using TaqMan MicroRNA Assays (assay IDs 000470, 000471 and 000475 respectively) according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). All reactions were performed in triplicate and run on an Applied Biosystems 7900HT Fast Real-Time PCR System. microRNA expression levels were normalized against mir-25, whose expression is unchanged with statin exposure (Figure S1). Statin-induced change in mir-148a, mir-148b and mir-152 was calculated using the delta delta Ct method. Paired two-side t-tests were used to identify differences in *HLA-G* and miRNA expression levels with statin treatment. *HLA-G* and microRNA expression levels quantified in LCLs were quantile normalized, split by rs1063320 genotype group, and tested for correlation using linear regression.

HLA-G protein quantitation

Soluble HLA-G protein levels were quantified by ELISA (BioVendor, Candler, NC) in cell culture media from *in vitro* 2.0 μ M simvastatin versus sham exposed LCLs. All measurements were performed in duplicate and averaged.

Genotyping

Donors of CAP LCLs were subject to genome-wide genotyping on the Illumina HumanHap300 or HumanHap610-Quad genotyping platforms (dbGaP accession number: phs000481), and rs1063320 was imputed as previously described[31]. Genotypes with an imputed probability less than 0.85 were omitted from the analyses. rs1063320 was directly genotyped with a TaqMan SNP Genotyping assay (Applied Biosystems).

Study Subjects

Study subjects were ascertained from the Personalized Medicine Research Project at the Marshfield Clinic in Wisconsin, population-based biobank. PMRP is comprised of 20,000 non-Hispanic whites of Northern European descent (98%) from Central Wisconsin. Enrollment was conducted in accordance with the guidelines established by the Institutional Review Boards of the Marshfield Clinic Research Foundation. Access and evaluation of the PMRP was performed through the Pharmacogenomics Discovery and Replication in Large Populations (PGPop) Consortium.

We identified individuals with asthma using ICD-9 codes (493*), with diagnoses indicated on two separate days. We have previously demonstrated that requiring the presence of such codes on two separate days substantially increases phenotypic accuracy (>95%) and replication for known genetic signals [34, 35]. Exclusion criteria for both cohorts included any individual with a single diagnosis code for chronic obstructive pulmonary disease (496), cystic fibrosis (277*), immunodeficiency (279*), bronchiectasis (494*), hereditary and degenerative diseases of CNS (331), mental retardation (317, 318, 319), congestive heart failure (428*, 429.9), pulmonary hypertension or embolism (415*, 416*, 417*), lupus (710.0), RA (714.0), tuberculosis (511.1), lung cancer (162*), or sarcoidosis (135). These algorithms were validated by manual chart review of 50–100 records per cohort and found to have >90% PPV.

Statin users were then identified as individuals with at least two mentions of statin drugs (simvastatin, fluvastatin, pravastatin, rosuvastatin, atorvastatin, lovastatin or any brand names of these drugs) of the same type and dose within a 6-month time window. We have previously demonstrated the capacity of natural language processing to document statin exposure since this type of data can generate accurate dose response curves despite the lack of information regarding drug compliance [36]. Statin users with asthma were defined as those with asthma drug use or diagnosis indicated after the date of first statin mention. From this analysis we identified 421 subjects (Tables S1 and S2).

Case definition

Asthma related exacerbations were defined as emergency department visits, hospitalizations, and/or use of oral steroids (prednisone, methylprednisone, solumedrol, solu-medrol, orapred, orasone, medrol, depo-medrol, depomedrol, depopred, prednisolone) for which an ICD-9 code for asthma or wheezing (786.07) appeared in the medical record concurrently. Since a high percentage of individuals cease statin use within the first year of prescription, to increase the likelihood that identified individuals were still on statin during the time of the exacerbation, we only included exacerbations that occurred within 6 months (+/- 180 days) of a statin mention in the EMR. This algorithm had a >90% PPV.

Covariate definition

All prescription information, as well as weight, height and smoking status was collected for evaluation as a potential confounding variable. Smoking status was divided into three categories: “current”, “former” or “never smoker”. Confirmed use was validated in a subset of charts by manual review. Subjects were classified as having inhaled corticosteroid (ICS)

use if they were prescribed beclomethasone, budesonide, budesonide/formoterol, ciclesonide, flunisolide, fluticasone, fluticasone/salmeterol, mometasone, mometasone/formoterol, triamcinolone.

Statistical Analyses

A general linear model was used to test for a relationship between rs1063320 and statin effects on number of exacerbations (treated as a continuous variable), and included adjustment for gender, smoking status and ICS use. All analyses were performed in JMP 9.1 (SAS Institute, Cary, NC) using an additive model.

RESULTS

Mir-148b and mir-152 are statin responsive and correlated to HLA-G in an rs1063320 dependent fashion

To determine if mir-148a, mir-148b or mir-152 transcript levels are statin responsive, we incubated both LCLs (n=48) and HepG2 cell lines (n=6 replicate experiments) with either 2.0 μ M simvastatin or sham buffer for 24 hours, and quantified changes in transcript levels using real-time PCR. Mir-148b and mir-152 were upregulated with statin treatment in both cell types (p<0.05, Figure 1A). No change in mir-148a levels was detected.

The rs1063320 C allele has been previously reported to disrupt the binding sites of mir-148 and mir-152 in the *HLA-G* 3' UTR[27]. Using previously collected genotype data for the LCLs, we confirmed that the C allele carriers had 1.3-fold greater *HLA-G* transcript (p<0.0001, N=160) than non-carriers (Figure 1B). Furthermore, we found that inter-individual variation in microRNA and *HLA-G* transcript levels was correlated in the LCLs in an rs1063320 dependent fashion. Specifically, lower expression levels of mir-148a and 152 were correlated with higher *HLA-G* transcript levels, but only in the LCLs derived from rs1063320 GG homozygotes (p<0.05, n=11). No correlation was seen in LCLs from either CC or CG rs1063220 donors (n=15, Figure 1C). A similar, non-statistically significant, trend was observed between mir-148b and *HLA-G*.

It has been previously suggested that the mir-152 family regulates HLA-G by impacting translation and not mRNA decay [28]. Thus, we quantified both *HLA-G* transcript and sHLA-G protein levels in LCLs after 24hr *in vitro* incubation with 2.0 μ M simvastatin or sham buffer. While we detected no change in *HLA-G* transcript levels in LCLs that carried the C allele, we found that statin treatment increased *HLA-G* transcripts levels (p=0.01, Figure 1B) in the GG homozygotes. In contrast, we observed a reduction in HLA-G protein levels in both genetic groups, but there was a trend (p=0.09) of greater reductions of HLA-G protein in the GG versus CC homozygotes (Figure S2). These findings are consistent with our hypothesis that rs1063320 modifies statin effects on *HLA-G* expression.

rs1063320 genotype is associated with frequency of exacerbations in statin users

To test our hypothesis that statins may benefit asthma in an rs1063320 dependent fashion, we used logistic regression with adjustment for significantly associated covariates (see Methods). Consistent with our hypothesis, we found that individuals who carried at least one

copy of the G minor allele had a reduced frequency of asthma-related exacerbations (emergency department visits, hospitalizations or oral steroid use) compared to non-carriers using an additive genetic model ($p=0.03$), Figure 2. There was no difference in exacerbation frequency between GG and CG genotypes.

DISCUSSION

We report evidence that statin treatment increases expression of mir-148b and mir-152, has no impact on HLA-G transcript in rs1063320 CC homozygotes, and appears to cause greater reductions in HLA-G protein levels in GG homozygotes. HLA-G expression is regulated by the mir-152 family of microRNAs (mir-148a, mir-148b and mir-152), and rs1063320 is thought to disrupt this interaction [27, 28, 38]. Since HLA-G levels are increased in asthmatic patients, our identification that statin treatment up-regulates mir-148b and mir-152 and influences HLA-G expression in a rs1063320 dependent manner lead to our hypothesis that statin treatment may show greater benefit in individuals with the rs1063320 G allele (Figure 3). Consistent with this hypothesis, we observed that asthmatic patients with at least one copy of the G allele had a reduced frequency of exacerbations while on statin compared to CC homozygotes. Given the immunomodulatory properties of HLA-G, it has been proposed that understanding gene regulation and the role of polymorphisms (especially those in the 3' UTR) in *HLA-G* may help inform the development of personalized medicine [39]. Specifically, rs1063320 is only one of two *HLA-G* SNPs proposed to have clinical potential, and assays have been developed for quick typing of this polymorphic site [40, 41].

Nicodemus-Johnson *et al* (2013) recently reported that asthmatic patients with the rs1063320 G allele had reduced sHLA-G in BAL fluid compared to non-carriers [28]. Consistent with this report, we also found that LCLs homozygous for the G allele had reduced sHLA-G protein levels compared to CC homozygotes. Interestingly, they did not identify a statistically significant association between the SNP and *HLA-G* transcript in the bronchial epithelial cells themselves. In addition, Zhu *et al* (2010) demonstrated that mir-152 overexpression caused reduced HLA-G protein levels by targeting the 3' UTR, but had no effect on *HLA-G* transcript levels [38], leading to the suggestion that the mir-152 family modulates HLA-G at the level of protein translation rather than mRNA decay. Here, we report disparate effects of statin treatment on HLA-G transcript versus protein levels in the rs1063320 GG homozygotes, consistent with the possibility that mir-148 and mir-152 may influence *HLA-G* translation. It is possible that the increase in *HLA-G* transcript levels in the GG homozygotes after statin treatment is due to a compensatory upregulation of gene transcription if statin treatment attenuates transcript translation. However, our identification of an association between rs1063320 and *HLA-G* transcript levels, along with the negative correlation observed between mir-148a, mir-148b and mir-152 expression levels with *HLA-G* transcripts, strongly suggest that these microRNAs modulate *HLA-G* transcript levels as well. Thus, further studies are required to ascertain the exact mechanism by which the mir-152 family regulates HLA-G.

Recently it has been called into question whether rs1063320 modifies mir-148a, mir-148b and/or mir-152 regulation of HLA-G. It has been independently reported that mir-148 and mir-152 overexpression reduces HLA-G by targeting its 3' UTR [38, 42], and that this

targeting impacts HLA-G function [42]. Although Tan *et al* (2007) originally reported that rs1063320 modulated luciferase expression from a *HLA-G* 3' UTR reporter construct in the context of mir-148a, mir-148b and mir-152 overexpression, Manaster *et al* (2012) failed to replicate this finding [27, 42]. Although our results are consistent with the possibility that rs1063320 directly impacts HLA-G regulation, we did not test it directly. Thus, it is possible that the associations observed in the LCLs between rs1063320 with HLA-G transcript and protein in both the statin and sham treated states are mediated by an alternate SNP in high linkage disequilibrium with rs1063320.

Statin use has been associated with reduced COPD related exacerbations as well as overall lower mortality [11, 43–45]. Although there is currently no literature supporting a link between COPD and *HLA-G*, levels of both mir-148a and mir-152 has been reported independently to be differentially expressed in subjects with COPD [46–48], suggesting that *HLA-G* may also play a role in COPD. Thus, it is possible that rs1063320 may modulate statin benefit in COPD as well.

Despite the strengths of our study, there are a number of caveats to our results. First, all subjects included in the study received statin treatment during the course of routine clinical care, and statin users were identified by natural language processing of the electronic medical records. Thus, we have no direct measures of drug compliance or pharmacy information. However, we did require that the same statin type and dose were mentioned twice in the EMR within a 6-month time window to increase the likelihood that individuals were dose-stabilized and drug compliant. Furthermore, we extensively validated our algorithms using chart review. We have previously used such techniques to identify genetic variation associated with LDL cholesterol response to statin treatment [36]. Second, although we were able to identify a statistically significant association between rs1063320 and asthma-related exacerbations in statin users, replication in independent cohorts is necessary to confirm this association. Notably, using the same algorithms described above, we identified 453 asthmatic statin users in BioVU, a population based biobank at the Vanderbilt University Medical Center in Tennessee. However, only 6 of the 453 subjects identified had a documented exacerbation after the start of statin use, and thus this cohort was severely underpowered to test for replication. Third, we were not able to control for all known covariates. For example, the relationship between rs1063320 and asthma status has been shown to be dependent on maternal asthma status, and we were unable to reliably extract this information from the EMR [27, 28]. In addition, smoking status is particularly challenging to evaluate based on health records alone [49].

Although the rs1063320 C allele is the ancestral allele, its frequency varies between populations: 45.6% in Northern Europeans (CEPH), 58.1% in Han Chinese, 65.5% in Yorubans and 73.3% in Japanese. Thus, if it is successfully validated that the C allele reduces statin benefits for asthma, given the high prevalence of this variant across the global population, these results could help explain discrepancies in studies of statin effects on the severity of these diseases. Importantly, such a relationship could identify a substantial number of asthmatics worldwide for whom statins may be a viable treatment alternative. However, the current results should be considered preliminary observations and are not intended to guide current statin prescription.

In summary, statins up-regulated mir-148b and mir-152, and affect HLA-G expression while modulated by rs1063320. Consistent with this mechanism, we observed that rs1063320 is associated with frequency of asthma-related exacerbations in statin users. These results provide support for genetically-influenced heterogeneity of treatment response to statins for individuals with asthma. Importantly, rs1063320 genotype could inform future statin use, thus introducing another therapeutic option for the management of asthma.

Supplementary Material

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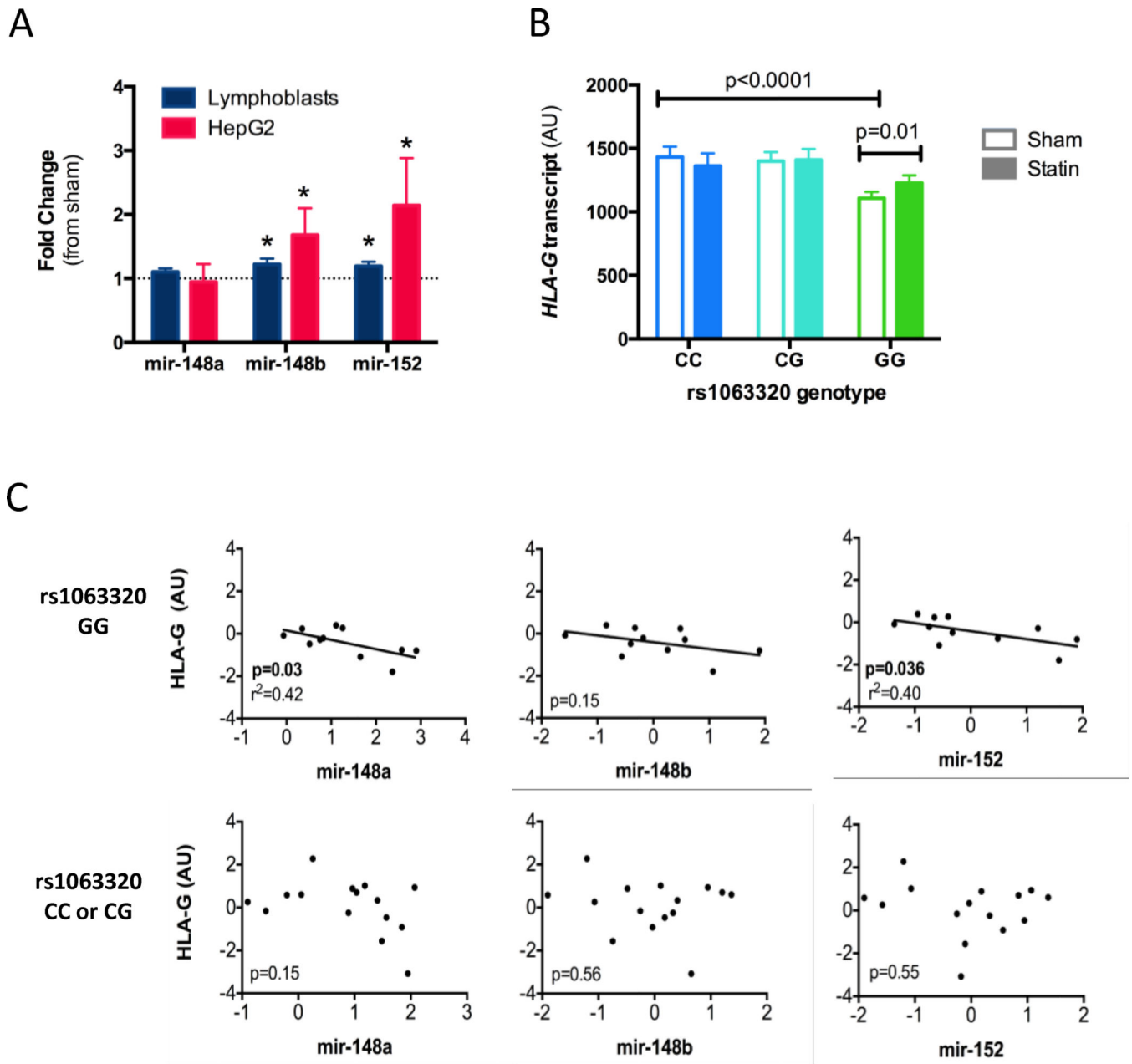


Figure 1. mir-152 family and HLA-G expression levels are statin responsive

A) CAP LCLs (n=48) and HepG2 (n=6 replicate experiments) cells were incubated with 2.0 μ M simvastatin or sham buffer for 24 hours, and mir-148a, 148b and 152 were quantified by qPCR, with values normalized against mir-25. Values shown are fold change (statin/sham values) \pm standard error, *p<0.05. **B)** Normalized *HLA-G* transcript level was quantified by expression array in statin or sham treated LCLs (CC n=32, CG n=42, GG n=86) as previously described[30] and tested for association with rs1063320 genotype using an additive model. **C)** Expression levels of *HLA-G* and the three microRNAs were measured in sham treated LCLs, quantile normalized, and tested for correlation across the samples split by rs1063320 genotype: GG n=11, CC or CG n=15.

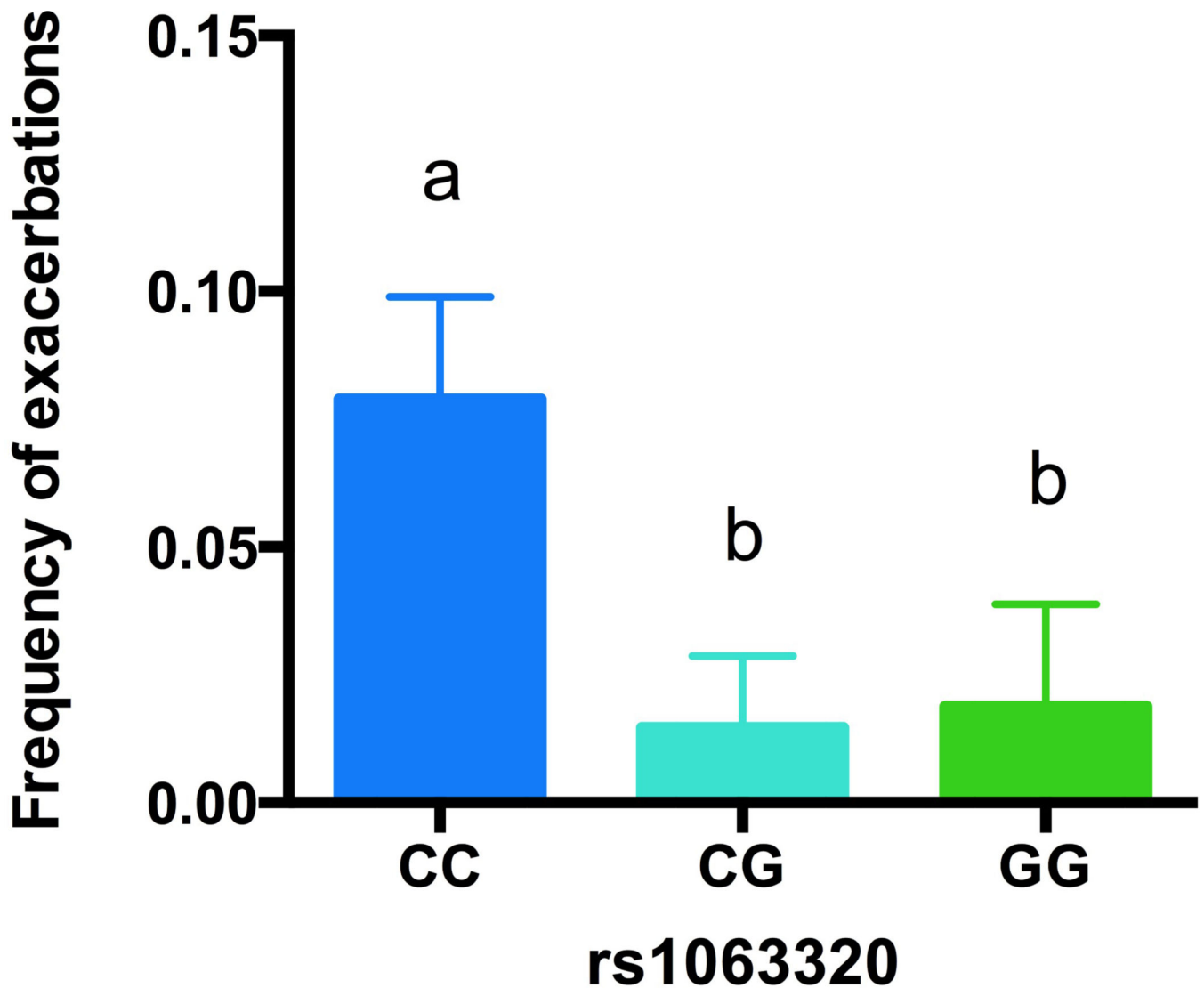


Figure 2. Association between rs1063320 and asthma-related exacerbations
rs1063320 genotype and frequency of asthma-related exacerbations in statin users was tested using an additive general linear model with adjustment for smoking status, gender and inhaled corticosteroid use ($p=0.03$). A post-hoc student's t-test was used to identify differences between genotypic groups as indicated by "a" or "b". CC $n=111$, CG $n=204$, GG $n=106$. Values shown are least square means \pm standard error.

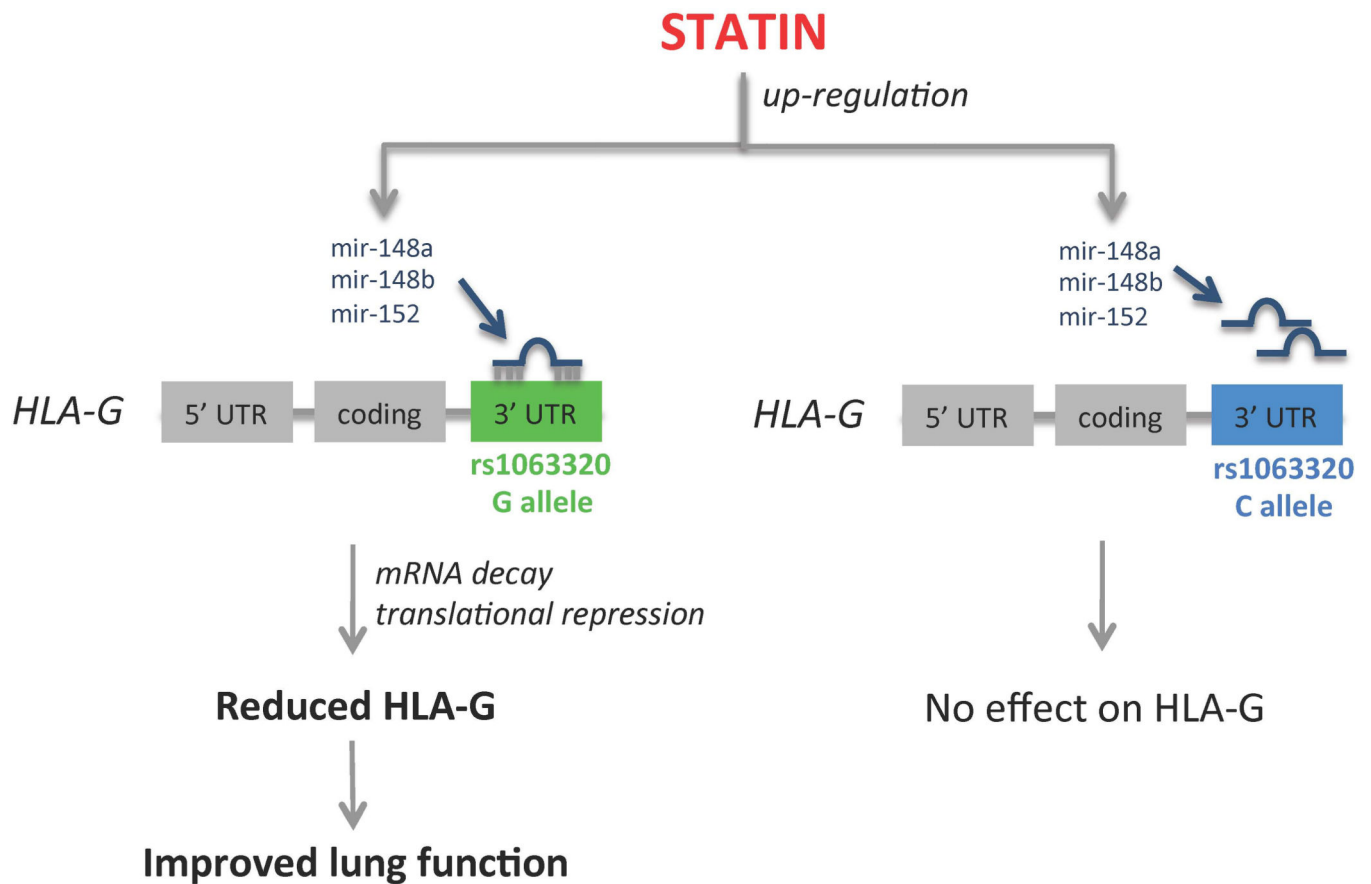


Figure 3. Hypothetical model

Statin treatment up-regulates mir-148b and mir-152, which target the *HLA-G* 3'UTR in an rs1063320 dependent fashion, leading to the prediction that statin treatment has greater benefit in individuals with asthma that carry the rs1063320 G allele.

Table 1

PMRP Subject characteristics

N	421
Age (median)	52
% Male	34.2
% Inhaled corticosteroid use	66.7
% Non-smoker (never)	51.4
% With exacerbation (1, 2 or 3)*	2.8
Statin Type**	
Simvastatin (%)	26.4
Lovastatin (%)	9.0
Pravastatin (%)	5.2
Fluvastatin (%)	3.5
Atorvastatin (%)	54.5
Rosuvastatin (%)	1.4

* Exacerbation defined as asthma-related ER visit, hospitalization or oral steroid use that occurred within 6 months of a statin mention in the EMR.

** Either type of statin listed just prior to exacerbation, or first known statin in those without an exacerbation within 6 months of the first statin mention.