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Investigating Genetic Predictors of Inhibitors among Persons with Hemophilia

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Investigating Genetic Predictors of Inhibitors among Persons with Hemophilia

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M.P.H., Emory University Rollins School of Public Health, 2011

B.S., Georgia Institute of Technology, 2007

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Abstract

Investigating Genetic Predictors of Inhibitors among Persons with Hemophilia

By Amanda B. Payne

Hemophilia A (HA), an inherited bleeding disorder affecting approximately 20,000 males in the United States, is caused by pathogenic variants in the *F8* gene leading to loss or reduced functionality of the procoagulant factor VIII (FVIII). HA is most-commonly treated by replacing the missing or dysfunctional FVIII. Unfortunately, 10%-15% of persons with HA develop antibodies (inhibitors) to the replacement FVIII, rendering it ineffective. Identifying persons at highest risk of developing inhibitors is important, as treatment may be altered. A previously-validated inhibitor-risk-prediction tool has limitations, including its reliance on prior hemophilia treatment and knowledge of family history. An inhibitor-risk-prediction tool that relies on information available at the time of HA diagnosis could be more useful clinically. Risk factors for inhibitors include situations that make recognition of foreign FVIII and upregulation of the immune system more likely. Family studies have indicated a genetic component to inhibitor risk. This dissertation explores the feasibility of constructing an inhibitor-risk-prediction tool that uses only genetic information.

In **Aim 1** information about the hemophilia genotype was used to predict inhibitor status. Three different paradigms to categorize hemophilia genotype were constructed, and the ability of each to predict inhibitor status was evaluated. The tool that used previously-published estimates of hemophilia genotype effect performed best; however, none of the tools performed as well as the previously-validated tool.

In **Aim 2** variation in immune response genes was used to predict inhibitor status. Estimates for the effect size of genetic variants were obtained in two ways: a meta-analysis was performed on published studies, and estimates were empirically derived from a genetic association study. Two tools were then developed, using estimates from the meta-analysis or the empirically-derived data. Neither of the tools performed as well as the previously-validated tool.

Aim 3 combined information from aims 1 and 2, using both hemophilia genotype and variation in immune response genes. The best-performing tool performed similarly to the previously-validated tool, without requiring treatment or family history information

The results of this investigation indicate that prediction of inhibitors using only genetic information may be possible. Further validation of the results in an external population is warranted.

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Table of Contents

Tables

Figures

Chapter 1: Introduction and Rationale

Hemophilia A, a genetic disorder caused by mutations in the *F8* gene on the X chromosome, leads to loss or reduced functionality of factor VIII – an important pro-coagulant protein that plays a role in the conversion of prothrombin to thrombin in the ultimate formation of the stable fibrin blood clot. Hemophilia is associated with bleeding that leads to increased risk of joint damage, intracranial hemorrhage, complications following procedures and surgeries, and severe bruising. The disorder occurs in approximately 1 in 5,000 male births in the United States each year and currently affects approximately 20,000 males in the United States.[1]

Hemophilia treatment involves the replacement of the missing or dysfunctional clotting protein. The use of factor replacement products is not without risk. Approximately 10%-15% of all persons with hemophilia will develop an antibody, known as an inhibitor, which attacks the treatment product, making it ineffective.[2] Inhibitors result in increased risk of bleeding[3] and early mortality[4], factor product utilization[3], and healthcare costs[5].

It has been hypothesized that inhibitor development is likely a function of at least three components, including environmental characteristics, patient genetics, and treatment characteristics.[6] There have been some attempts the develop risk stratification tools to identify persons with hemophilia at increased risk for inhibitor development based on these risk factors.[7] However, some[8] have criticized that current risk stratification tools are not ideal because they require exposure to factor product and knowledge of family history and argue that a score based on genetic markers would be more useful in the clinical setting.

There are at least two important genetic components related to inhibitor development: the hemophilia genotype and drivers of immune response. It is understood that the hemophilia genotype is a major predictor of inhibitor risk, with large-impact hemophilia-causing variants having the highest risk or inhibitor development, and low-impact hemophilia-causing variants having the smallest risk.[9] However, it has been estimated that among those with low-impact

variants, the risk of inhibitor development is not zero.[10] This indicates better classification of hemophilia-causing variants may improve inhibitor prediction. It is also understood that, at some level, inhibitor development is an immune response complication.[6] Inhibitor development likely involves several cell types and proteins involved in immune response, including the major histocompatibility complex (MHC) and cytokines.[6] Genetic variation in either the MHC region or in genes that code for cytokines and their receptors could affect inhibitor risk.

The Aims of this dissertation project center around evaluating the feasibility and utility of developing an inhibitor risk stratification tool that relies heavily on genetic information. The two genetic components of the tool are: 1) information regarding the hemophilia genotype and 2) information regarding variation in genes in the immune response pathway.

Aim 1 explores the hemophilia-causing genotype and its relationship to inhibitor risk among persons with hemophilia A. The ability of several hemophilia-causing variant classification tools to correctly assign inhibitor status in a cohort of patients with hemophilia A with and without inhibitors is evaluated, with the goal of finding the tool that most accurately predicts inhibitor status. These results will be used to inform the first component of the risk prediction tool.

Aim 2 explores the relationship between variation in genes in the immune response pathway and inhibitors among persons with hemophilia. The results of a systematic review and meta-analysis of available data regarding associations between variants in genes in the immune response pathway an inhibitors among persons with hemophilia A are presented as well as the results of an analysis examining the association between variants in genes in the immune response pathway and inhibitors among persons with hemophilia A in a large study of inhibitors conducted in the United States. These results are used to inform the second component of the risk prediction tool.

Aim 3 combines information from Aims 1 and 2 to produce a series of inhibitor risk prediction tools. The ability of each tool to correctly assign inhibitor status in a cohort of patients with

hemophilia A with and without inhibitors is evaluated. The performance of each tool is also compared to a previously-validated tool that includes non-genetic risk factors for inhibitor development.

Chapter 2: Background

Hemophilia A

Hemophilia A (HA) is an inherited bleeding disorder that occurs in 1 in 5,000 male births and affects approximately 20,000 males in the United States.[1] HA is an X-linked recessive disorder caused by pathogenic variation in the *F8* gene that leads to loss or reduced functionality of factor VIII (FVIII).[11] FVIII is a pro-coagulant protein that interacts with factor IX in the presence of calcium ions and phospholipids to form a complex that converts factor X to activated factor X.[12] Activated factor X converts prothrombin to thrombin, which in turn converts fibrinogen to fibrin to form a stable blood clot.[12] FVIII is protected in circulation from proteolytic cleavage by von Willebrand factor.[12] The loss or reduced functionality of FVIII associated with HA causes bleeding episodes that can lead to increased risk of intracranial hemorrhage, joint damage, severe bruising, and complications following surgery.[11,13,14] The severity of the phenotype is closely correlated with the FVIII activity level (FVIII:C), with severe disease being associated with <1% FVIII:C, moderate disease being associated with 1% ≤ FVIII:C ≤ 5%, and mild disease being associated with >5% FVIII:C.[15]

Inhibitors in Hemophilia A

HA is treated by replacing the missing or defective protein using either plasma-derived FVIII from donated blood or FVIII manufactured using recombinant technologies.[16] A subset of persons with HA will develop antibodies directed against the treatment product.[16,17] Inhibitory and non-inhibitory antibodies can develop.[18,19] Inhibitory antibodies (inhibitors), which render treatment products ineffective, are directed against FVIII epitopes that play a role in FVIII function, such as interaction sites with factor IX, phospholipids, or von Willebrand factor.[20-22] It has been estimated that inhibitors occur in up to 30% of persons with severe HA and 3-13% of persons with mild/moderate HA.[2,23-27] Among persons with severe HA, the risk of inhibitors is highest during the first few exposure days to treatment, with peak inhibitor development usually

occurring after 10-14 exposure days then leveling off at a lower rate after 150 exposure days.[28-30] In contrast, the risk among persons with mild/moderate HA, while initially lower than that of those with severe HA, continues to rise with increasing number of exposure days throughout the lifespan.[31] This leads to a bimodal distribution with respect to age, with a peak in early age groups and another peak in older age groups.[28,32]

Impact of Inhibitors

Inhibitors are associated with increased risk of bleeding[3], disability[33], early mortality[4,25], healthcare costs[3,5,34-40], and decreased quality of life[41]. Because inhibitors render treatment products ineffective, more-costly and less-effective bypassing agents are used to control bleeding episodes.[35-38,42,43] The half-life of traditional bypassing agents, such as recombinant factor VII, is approximately 2.5 hours[44], compared to 8-12 hours for traditional FVIII products[45]. These leads to a reduced capacity to control and prevent bleeding episodes. A 2014 report by Armstrong et al reported that the annual bleeding rate among persons with inhibitors was over 4 times greater than that among persons without inhibitors.[3] Furthermore, the use of bypassing agents to treat and prevent bleeding episodes is considerably more expensive than traditional treatment, with the average annual costs among persons with inhibitors being 4 times that of persons without inhibitors.[34,40]

How Inhibitors Develop

Inhibitor development is a T cell mediated process.[6,46] Foreign FVIII binds to surface immunoglobulin on the surface of antigen presenting cells (APCs).[6,46] The bound FVIII is endocytosed and proteolytically cleaved by the APC.[12,34,47] The resulting peptides are bound to class II major histocompatibility complex (MHC) molecules and transited to the APC surface where the peptide is presented to T cell receptors on CD4 lymphocytes.[47,48] A second interaction between the APC and the T cell through CD80/86 molecules on the APC and CD28 molecules on

the T cell is required for proper presentation of the antigen to the T cell receptor, thereby activating the T cell.[49,50] T cell activation, the release of cytokines, and the upregulation of several proteins on the surface of the CD4 lymphocyte promotes the interaction between the T cell and B lymphocytes, thereby promoting B cell proliferation and differentiation into antibody-secreting plasma cells.[51]

Risk Factors for Inhibitor Development

Risk factors for inhibitor development include situations that would make antigen presentation and immune system response more likely.[6,46,52]

Non-Genetic Risk Factors

Studies indicating discordant inhibitor phenotypes among monozygotic twins highlights the likelihood of a non-genetic component to inhibitor risk.[53,54] As inhibitor development is most likely to occur within the first 10-14 exposure days among persons with severe HA when exogenous FVIII is most likely to be recognized as foreign, age is a known risk factor for inhibitor development – with younger age groups being at highest risk.[55,56] Furthermore, intense treatment at the first exposure has been shown to be associated with increased risk.[30,57-59] Conversely, low-dose, regular treatment (i.e., prophylactic treatment) has been shown in some studies to be protective against inhibitor development.[30,60,61] It has been hypothesized that intense treatment can lead to cell injury or inflammation that leads to immunologic 'danger signals' that can stimulate APCs and amplify the immune response.[62-64] The type of treatment product used at initiation of treatment also appears to be important, with recombinant FVIII products shown to be associated with an 87% higher incidence of inhibitors compared to plasma-derived FVIII among previously untreated patients in a randomized trial.[65]

Genetic Risk Factors

While family studies suggest a likely environmental component to inhibitor risk, they also suggest a strong genetic component. The Malmo International Brother Study (MIBS) found an overall concordance of 78.3% with respect to inhibitor phenotype among enrolled brothers pairs.[53] Furthermore, the risk of inhibitors in families with a known family history of inhibitor development was 48%, over 3 times the risk in families with no family history (15%).[30] Genetic risk factors for inhibitor development include the hemophilia-causing genotype and variation in genes involved in immune response.

Hemophilia Genotype

HA is caused by pathogenic variation in *F8*, a 26-exon gene that codes for the 2,332-amino-acid FVIII protein.[11] The FVIII protein is split into 2 chains.[66] The heavy chain contains domains A1, A2, and B.[66] The light chain contains domains A3, C1, and C2.[66] The A1 domain participates in FX binding.[66] The A2 domain participates in FIX binding.[66] The B domain is proteolytically cleaved and is relatively dispensable for FVIII activity.[66] The A3 and C2 domains participate in binding to von Willebrand factor.[66] Approximately 45% of severe HA is caused by 1 of 3 gene inversions: an inversion between a repetitive region in intron 1 and a similar region 140 kb 5' to *F8* (intron 1 inversion), an inversion between a repetitive region in intron 22 and a similar region 300 kb 5' to *F8* (intron 22 type 2 inversion), or an inversion between a repetitive region in intron 22 and a similar region 400 kb 5' to *F8* (intron 22 type 1 inversion).[67,68] While most with a gene inversion will exhibit the severe HA phenotype, inversions have been reported in up to 3% of persons with moderate disease.[69] Furthermore, it has been shown that large polypeptide chains, though not full-length FVIII, can be synthesized in inversion-transfected cells.[70] The remaining 55% of severe HA and vast majority of mild/moderate HA is caused by pathogenic variation elsewhere in *F8*. Over 2,500 variants have been reported to cause HA.[71] The most common non-inversion variants among persons with severe HA are those resulting in

a single amino acid substitution in the amino acid sequence (missense variants – 17%) or single-nucleotide insertions or deletions that alter the reading frame and subsequently result in early termination of the protein sequence (frameshift variants – 17%).[69] Less common are single-nucleotide switches that result in an early termination of the protein sequence (nonsense – 11%), large deletions or duplications encompassing more than 50 nucleotides (6%), variants that alter exon splicing (splice site – 3%), and multi-nucleotide insertions or deletions that do not alter the reading frame (1%).[69] Among persons with mild/moderate disease missense variants are the most common non-inversion variants (80%), followed by single-nucleotide switches that do not result in changes to the amino acid sequence (6%), splice site (3%), frameshift (2%), and multi-nucleotide insertions or deletions that do not alter the reading frame (0.4%). Up to 1% of persons with severe HA and 3% of persons with mild/moderate HA have no identifiable variant.[69]

The risk of inhibitor has been associated with the hemophilia-causing genotype in multiple studies.[10,31,72-74] Variants that are likely to interfere with protein production are particularly immunogenic. A meta-analysis investigating the relationship between the hemophilia-causing variant and inhibitor development among persons with severe HA indicated that, compared to persons with intron 22 inversions, persons with large deletions were 3.6 times more likely to be inhibitor-positive, persons with nonsense variants were 1.4 times more likely to be inhibitor-positive, and persons with missense variants were 70% less likely to be inhibitor-positive.[10] Furthermore, a recent investigation of 231 persons with severe HA indicated that participants with variants predicted by in-silico analyses to produce no protein product were twice as likely to develop inhibitors than participants with variants that were predicted to produce some protein product. Participants who were antigen-negative (indicating no protein product production) were 3.5 times more likely to develop inhibitors than participants who were antigen-positive.[75] The location of the variant also appears to be important. Among a large cohort of persons with mild/moderate HA, 19 of 214 missense variants were identified in at least

one inhibitor-positive participant. Of the variants seen in at least 10 participants, variants associated with the highest risk of inhibitor development were in the A1, C1, or C2 domains.[31]

Immune Response Genetics

Variation in genes involved in the immune response pathway has also been linked to inhibitor risk. Several groups have investigated the association between variants in genes involved in immune response and inhibitors among persons with HA.[59,76-112] However, the degree to of consistency in methods and results among the various investigations is unclear. For example, Astermark et al[100] reported an association between variants in *IL10*, a gene that codes for a protein that plays an important role in limiting the immune response, and inhibitors among persons with HA, but Bafunno et al[103], Lozier et al[79], and Pavlova et al[84] did not replicate this association. Variation in genes that code for the proteins involved in immune response could increase or decrease protein function, thereby promoting or limiting the likelihood of immune response. Variation may also play a role in the propensity for foreign antigen recognition. For example, each class II MHC molecule recognizes a unique repertoire of peptide sequences.[113] Certain class II MHC variants are more promiscuous than others, recognizing a wider repertoire of protein sequences. Persons with these variants may be more likely to develop inhibitors than others. In a large-scale computational study of inhibitor risk, Shepherd et al reported that certain *HLA-DRB1* variants recognized a wider variety of peptide sequences than others (e.g., *HLA-DRB1**01:01 was predicted to recognize a wider variety of peptide sequences than *HLA-DRB1**01:03).[114] Finally, variations in genes involved in the immune response pathway may not directly influence gene function but may be markers of alleles that do. Because segments of a chromosome that are close together tend to be inherited together[115], it could be that variants outside the coding region are genetically linked with more important variants within the coding region or in regulatory regions of the gene. Fine mapping of genes in the immune response pathway could identify regions of genes that are important in inhibitor development.[79]

Race/Ethnicity

In addition to treatment-related and genetic risk factors for inhibitor development, race/ethnicity has also been identified as a risk factor for inhibitor development.[53,59,116,117] Blacks and Hispanics have a nearly 2-fold increased risk of inhibitor development compared to Whites.[59,117] The reason for this is unclear. Viel et al proposed the difference in risk could be explained by a mismatch between the *F8* sequence commonly seen in almost exclusively Blacks and Hispanics and the sequence of recombinant FVIII most frequently used for treatment.[118] They reported a 3.6-fold increased prevalence of inhibitors among participants with this rarer sequence.[118] However, critics of this investigation cite concerns about sample size adequacy, appropriate control for confounding, and how the hemophilia genotyping was conducted, as hemophilia genotype was not determined for all study enrollees as was, instead, imputed.[119-123] A subsequent investigation by Gunasekera et al used three different approaches to evaluate the relationship between *F8* sequence mismatch and inhibitors.[124] The first was a larger case-control study that showed no correlation between inhibitor status and rare sequence.[124] The second was an investigation of binding affinities of peptides containing the relevant sequence mismatches that indicated weak or no binding in 85% of assays, indicating a low likelihood that the immune system would detect these sequences as foreign.[124] The third was as examination of cultured CD4 T cells from patients with mismatched *F8* and factor product sequences that indicated no reactivity of cells to sequence mismatch.[124] Based on these results it seems unlikely that sequence mismatch could explain the difference in inhibitor risk seen among Blacks and Hispanics as proposed by Viel et al.[125] Other hypotheses, such as differences in the frequencies of genetic variants that may influence immune response function, have not been formally tested.

Predicting Inhibitor Risk

Only one tool to predict persons with HA most likely to develop inhibitors has been validated.[7] This tool, developed by the Concerted Action on Neutralizing Antibodies in patients with severe

HA (CANAL) study investigators, is based on scoring of three components: 2 points for a positive family history of inhibitor development; 2 points for having a high-risk hemophilia-causing variant; and 3 points for intensive treatment at the first product exposure.[7] In a validation study, inhibitor incidence was shown to increase with increasing point totals. Inhibitor incidence among those with no points was 6, rose to 23% among those with two points, and to 57% among those with 3 points or more.[7] The tool showed good discriminative ability, with an area under the receiver operating curve of 0.74.[7] While the CANAL investigators indicated the tool performed equally well in an external population, Hashemi et al report underestimation of risk in the "low-risk" category and overestimation of risk in the "high risk" category when they evaluated the performance of the tool in a different population of persons with severe HA.[126]

While the CANAL tool showed good performance, it has been validated only among persons with severe HA. Furthermore, its reliance on factor product exposure has been cited as a drawback, as this limits the ability to guide a physician as to whether exposure should be avoided.[8] The tool's reliance on family history has also been cited as a drawback, as 30% of persons with HA will have no family history of hemophilia.[8,11]

A reliable tool that could predict inhibitor risk among persons with severe as well as mild/moderate HA would be valuable. Identification of persons at high risk for inhibitor development could alert clinicians to investigate alternative treatment strategies that could reduce risk, such as avoiding intensive therapy, avoiding recombinant factor products during early exposure days, initiating early prophylaxis, or delaying exposure to FVIII by using other treatment products. As previously discussed, intensive therapy may cause "danger signals" that may upregulate the immune response to foreign FVIII.[62-64] Understanding which patients may be "primed" to elicit such immune upregulation could help guide clinicians when deciding the type of therapy to use to control bleeding. Similarly, because use of recombinant FVIII products

during the first exposure days has been associated with inhibitor development, understanding which patients may be most likely to elicit a response could help guide factor product choice.[65] Finally, among patients with the highest risk of inhibitor development, avoiding the use of FVIII may be a valid treatment option.[127] For example, among persons with mild/moderate disease with hemophilia-causing variants known to be particularly immunogenic, the use of desmopressin, a drug that causes the release of endogenous FVIII, has been shown to be effective in controlling bleeding episodes.[128]

Dissertation Aims

Given the limitations of the current risk prediction tool, this dissertation project will attempt to develop a risk prediction tool for use across all hemophilia severity types and, by requiring no information about family history and prior treatment with FVIII, could be more useful clinically. Genetic information, including the hemophilia genotype and information on a limited set of variants in the immune response pathway, could be available before the initiation of treatment as part of the diagnostic workup. This dissertation project will explore ways to maximize this information in order to predict inhibitor risk. Aim 1 will explore the hemophilia genotype and will evaluate different methods of categorizing hemophilia-causing variants with the goal of predicting inhibitor risk. Aim 2 will explore the role of variation in immune response in predicting inhibitor risk. Because little is known about the degree of consistency in methodology and results among reports of associations between variants in genes in the immune response pathway and inhibitors, a systematic review and meta-analysis will attempt to summarize the data and provide summary estimates of effect. Also, an analysis using gene mapping will attempt to identify novel loci that may influence inhibitor risk. Finally, Aim 3 will attempt to develop a risk prediction tool that incorporates both hemophilia genotype and immune-response-variant genotype information into an inhibitor risk prediction tool that is based on genetic information only. The performance of the tool will be evaluated by

investigating the ability to correctly assign inhibitor status in a cohort of persons with HA in which the inhibitor status is known.

Chapter 3: Evaluation of variant-scoring tools for use in assigning inhibitor risk among persons with hemophilia A

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Abstract

Development of inhibitory antibodies against factor replacement products is the most concerning public health problem facing persons with hemophilia (PWH). Although the genetic variant causing an individual's hemophilia is the best-characterized risk factor for inhibitor development, there is only one validated tool available to predict whether PWH A, with one of over 2,000 different disease-causing variants, are at increased risk of inhibitor development, and this tool has only been validated among persons with severe disease. To evaluate methods of scoring disease-causing variants three different variant-scoring tools were applied to 758 PWH A enrolled in the multi-center Hemophilia Inhibitor Research Study. Tools included a score based on American College of Medical Genetics (ACMG) guidelines for determining pathogenicity (pathogenicity tool), a score based on predicted functional implications of the variant (function tool), and a score developed using severity-specific externally-produced estimates of effect (evidence-based tool). The pathogenicity score was determined by assigning point values to each of the components outlined in the ACMG guidelines. The function score was assigned using the following criteria: 4 points for variants predicted to produce no protein product (multi-exon deletions), 3 points for variants predicted to produce a possible protein

product (inversions, single-exon deletions, large duplications, nonsense variants, splice-altering variants, and frameshift variants), 2 points for predicted immunogenic gene products (missense variants at known inhibitor epitope locations), and 1 point for missense variants outside of inhibitor epitope locations. The evidence-based score was assigned using log-odds-ratio values for specific variant categories from either a previously-published systematic review (for persons with severe disease) or using previously-published data from the INSIGHT cohort (for persons with mild/moderate disease). Tools were evaluated by comparing the score distributions among persons with and without an inhibitor using the non-parametric Wilcoxon rank sum test and by comparing the area under the receiver-operating curve (AUC). Among the 3 tools evaluated, the evidence-based tool performed best (AUC=0.6712 and AUC=0.6050 for persons with mild/moderate and severe disease, respectively). However, none of these tools performed particularly well, indicating risk stratification based on categorizing the disease-causing variant alone may not be effective. These tools attempted to stratify inhibitor risk based on only one aspect of inhibitor-development etiology. Tools that include other aspects of the etiology would likely perform better.

Introduction

Hemophilia A (HA), an X-linked inherited bleeding disorder, affects approximately 20,000 males in the United States.[1] HA is caused by defects in the *F8* gene that lead to missing or defective factor VIII (FVIII), a pro-coagulant protein necessary for normal hemostasis. It is most commonly treated by replacing the missing or defect protein with either plasma-derived or recombinant FVIII. Unfortunately, a subset of persons with HA (PWH) will develop antibodies (inhibitors) to the replacement FVIII, rendering the therapeutic ineffective. It has been estimated that this occurs in up to 15% of all PWH at some point during their lifetime.[2] Inhibitor development has been associated with increased frequency of bleeding[3], factor product utilization[3], healthcare costs[3,5,39], and risk of early mortality[4].

Understanding which patients may be most likely to develop inhibitors is important because it may help guide treatment decisions. Intensive factor replacement therapy may cause "danger signals" that upregulate the immune response to foreign FVIII.[62-64] Furthermore, use of recombinant FVIII products during the first exposure days has been associated with inhibitor development.[65] Understanding which patients may be "primed" to elicit an immune response could help guide clinicians when deciding the type of therapy to use to control bleeding.

The only validated risk stratification tool to predict which PWH may be most likely to develop inhibitors is based on family history of inhibitor development, type of HA-causing genetic defect, and intensity of first treatment product exposure.[7,126] However, this scores may be of limited utility because it relies on prior exposure to factor product.[8] Risk stratification tools that rely on genetics alone may be more valuable, as decisions regarding treatment can be made before the first exposure to factor replacement products.[8,129] Furthermore this score has been validated among severe PWH only, leading to questions about performance in non-severe PWH.[7,126]

One of the most well studied genetic risk factors for inhibitor development is the HA-causing genetic variant itself. There have been more than 2,500 unique genetic variants reported to cause HA.[71] Disease severity is correlated with variant type.[9,71] Among those with severe HA the most common genetic defects are gene inversions, while among those with mild and moderate HA the most common genetic defects are missense changes that lead to substitutions for one amino acid in the FVIII peptide chain.[9,71] Among those with severe HA, genetic defects predicted to cause a complete loss of factor VIII protein production have been associated with highest risk of inhibitor development.[10] On the other hand, among those with mild and moderate HA only a few specific genetic missense variants or missense variants that occur in specific domains of F8 gene have been associated with increased risk of inhibitor development.[31]

The possible utility of a risk stratification tool based solely on categorizing HA-causing genetic defects across all severity subgroups has not been established. The purpose of this study was to evaluate different categorization schemes in development of a risk stratification tool and to assess whether or not such a tool could be useful clinically to identify PWH at highest risk of inhibitor development.

Methods

Population

Data from participants enrolled in the Hemophilia Inhibitor Research Study (HIRS) were used for this analysis. The HIRS methodology has been previously described.[130] Briefly, PWH receiving care at 17 participating hemophilia treatment centers were enrolled in HIRS in order to determine the feasibility of conducting national surveillance for inhibitors and to identify potential risk factors for inhibitor development. Demographic, treatment and inhibitor history data were collected by a study coordinator from clinic records using a standard data collection tool. Participants underwent baseline and annual inhibitor testing and submitted blood specimens for genotyping. For this analysis, data from participants with HA were analyzed. Data from participants who refused genotyping or whose blood samples lacked sufficient DNA for genotyping were excluded. When relatives were co-enrolled in the study, data from the first enrolled relative was used. In order to control for the potential impact of race and due to a relatively small sample size, participants with race/ethnicity other than White, Non-Hispanic were excluded.

Inhibitor status was determined based on historical data and on inhibitor testing results during the course of the study. We considered a participant to have a history of inhibitor development if he was reported to have a measureable inhibitor titer of ≥1.0 Bethesda Unit (BU) on at least two occasions, or one measured inhibitor titer of ≥1.0 BU followed by the institution of immune tolerance induction therapy, or was confirmed inhibitor-positive using a modified Nijmegen Bethesda assay[131] by the centralized HIRS laboratory during the study period.

Genotyping

The hemophilia-causing genetic defect was determined as previously described.[9] Briefly, sequencing of the 5' untranslated region, all exons, intron-exon junction regions, and the 3' untranslated region of F8 was performed in forward and reverse directions using an automated analyzer (3730 DNA Analyzer, Applied Biosystems, Carlsbad, CA, USA) and the VariantSEQr™ protocol. Inversions of *F8* were detected using PCR.[67,68] Large duplications were identified using Multiplex Ligation-dependent Probe Amplification™ (MLPA)[132] using SALSA MLPA Kits P178-A1 Factor VIII (MRC Holland, Amsterdam, The Netherlands).

Risk Prediction Tools

Three different risk prediction tools based on the hemophilia-causing genotype were evaluated. A description of each tool is provided below. Variants were scored using the criteria for each tool.

Tool based on predicted pathogenicity (Pathogenicity Tool)

The American College of Medical Genetics (ACMG) has published guidelines for determining pathogenicity of genetic variants.[133] The guidelines outline how to categorize genetic variants as pathogenic, likely pathogenic, unknown significance, likely benign, and benign based on various pieces of evidence, including allele frequency in normal populations and predicted impact on gene function. Pieces of evidence were graded as 'very strong', 'strong', 'moderate', and 'supporting'. For this analysis each piece of evidence was assigned a point value. Points were summed across all evidence categories in order to produce a final variant score. The operationalization of each of the evidence categories is outlined in Supplementary Table 1. Participants with no HA-causing variant identified were not assigned a score.

Tool based on predicted impact on gene function (Function Tool)

A score based on predicted impact of each variant on protein function was assigned using the following criteria: 4 points for variants predicted to produce no gene product (multi-exon deletions)[134]; 3 points for variants predicted to produce a possible gene product (inversions, single-exon deletions, large duplications, nonsense variants, splice-altering variants, and

frameshift variants)[67,70,135-161]; 2 points for predicted immunogenic missense variants (missense variants at known epitope locations); and 1 point for missense variants outside of known inhibitor epitope locations. Epitope locations were identified by searching for antibody epitopes reported in the Immune Epitope Database.[162] Participants with HA-causing variants identified as in-frame deletions less than 50 base-pairs in length or synonymous variation or with no HA-causing variant identified were not assigned a function score.

Tool based on prior evidence (Evidence-Based Tool)

A severity-specific score based on previously-published estimates of effect of hemophilia-causing genetic variant types on inhibitor risk was assigned. For participants with severe HA the log-odds-ratio value for HA-causing variant types reported in a meta-analysis[10] was assigned as the evidence-based score. Scores for each variant category were assigned as follows: multi-exon deletions (OR 9.24), single-exon deletions (OR 1.09), nonsense variants occurring in the light chain of *F8* (OR 1.80), nonsense variants occurring outside the light chain (OR 1.04), intron 22 inversions (reference group – OR 1.00), intron 1 inversions (OR 0.92), small deletions within poly-A-runs (OR 0.27), small deletions outside poly-A-runs (OR 0.65), missense variants occurring in the light chain (OR 0.37), missense variants occurring outside the light chain (OR 0.23), splice site variants (OR 0.95), and no variant identified (OR 0.37). Participants with severe disease with HA-causing variants identified as complex gene rearrangements or with synonymous changes were not assigned an evidence-based score. For participants with mild or moderate HA the log-odds-ratio value for HA-causing variant types reported from the INSIGHT cohort[31] was assigned as the evidence-based score. Variant categories included missense variants not in the light chain of *F8* and not identified as high-risk (i.e. not p.Arg612Cys, p.Tyr2124Cys, or p.Arg2169His; reference group – OR 1.00), missense variants in the light chain and not identified as high-risk (OR 5.74), high-risk variants (OR 17.27), and all other variant types (OR 2.99). Participants with mild or moderate disease that did not have a HA-causing variant identified were not assigned an evidence-based score.

Statistical Analysis

Differences in variant scores between inhibitor-positive and inhibitor-negative participants were compared using the Wilcoxon rank sum test. The ability of a tool to correctly classify inhibitor status was evaluated using the area under the receiver operating curve (AUC) using a logistic model with history of inhibitor as the outcome and the score as the predictor. Performance was evaluated overall and by HA severity. All analyses were done using SAS, version 9.4 (SAS Institute, Cary, NC, USA).

Results

Among 1,300 participants in HIRS, 758 were eligible for this analysis, after exclusions based on hemophilia type (N=233), race/ethnicity (N=236), lack of genotyping data (N=34), and co-enrollment of a relative (N=39). Of these, 162 were inhibitor-positive, with 14 participants developing an inhibitor during the study period. HA-causing *F8* variants were identified in 739 participants. There were 306 unique variants identified. A summary of variant types by severity and inhibitor status is provided in Table 1.

Pathogenicity Tool

Pathogenicity scores did not differ significantly by inhibitor status (Figure 1A). When stratified by severity, there was also no significant difference in pathogenicity scores by inhibitor status. Furthermore, the AUC was not above 0.6, either overall or when stratified by disease severity (Figure 2).

Function Tool

The distribution of function scores in the overall study population and stratified by severity and inhibitor status is outlined in Figure 1B. Scores differed statistically significantly overall ($p<0.01$) and among participants with severe disease ($p<0.01$); however, as can be seen in Figure 3, these differences did not produce appreciably higher AUC (AUC=0.65 overall, AUC=0.52 among mild or moderate participants, AUC=0.58 among severe participants).

Evidence-based Tool

There are statistically significant differences in evidence-based scores when stratified by severity (Figure 1C). Because scores were assigned based on severity, distributions of the scores overall are not meaningful. The scores among inhibitor-positive participants with mild or moderate disease were higher than the scores among inhibitor-negative participants with mild or moderate disease ($p<0.01$). Similarly, the scores among inhibitor-positive participants with severe disease were higher than the scores among inhibitor-negative participants with severe disease ($p<0.01$). This is reflected in the AUC (Figure 4). The AUC among mild or moderate participants was 0.67, and the AUC among severe participants was 0.61.

Discussion

Inhibitor development is the most significant treatment-related complication among PWH. The ability to identify those at highest risk of inhibitor development early in their treatment experience is important, as treatment may be altered to avoid intensive factor product exposure, a strong environmental risk factor for inhibitors. A Previously-developed inhibitor risk prediction tool[7,126] has been validated only in subpopulations (e.g., among severe PWH) and requires information obtained only after prior product exposures. A tool based on patient characteristics alone would be more useful in the clinical setting, especially when faced with a situation that requires a decision on treatment.

The tools evaluated in this analysis utilized information about hemophilia severity and the disease-causing genetic variant – information that could be available prior to the need for treatment. Unfortunately, none of these tools performed particularly well, as evidenced by low predictive ability either overall or when stratified by hemophilia severity.

Each of the tools evaluated in this analysis were limited in some way. The pathogenicity tool was developed based on ACMG guidelines[133] for describing variant pathogenicity in terms of causing hemophilia. It is not surprising then that this tool was of limited utility in determining whether a variant may be immunogenic. Null variants that would likely be more immunogenic

and non-null variants could have similar pathogenicity scores, as they would both be predicted to cause hemophilia. Furthermore, the ability to determine pathogenicity of variants related to non-Mendelian outcomes, such as inhibitors, is listed as a limitation of the guidelines. The function tool was developed to avoid this limitation, as null variants were scored differently than non-null variants on an arbitrary scale. While this resulted in a score that differed between inhibitor-positive and inhibitor-negative participants, these differences were minimal and did not allow better prediction of inhibitor status based on score value. This may be related to the arbitrary nature the point values were assigned. The evidence-based tool attempted to assign values to variant categories based on prior evidence, rather than use of an arbitrary scale. Previously-published estimates of the effect of variant categories on inhibitor risk among severe[10] and mild/moderate[31] PWH were used to assign the evidence-based score in a severity-specific manner. This resulted in the best performing score. However, the score did not perform as well as the previously-published risk prediction tool[7,126] that incorporated other patient and treatment characteristics such as family history of inhibitor development, type of HA-causing genetic defect, and intensity of first treatment product exposure.

Inhibitor development is theorized to be a T-cell dependent process that involves the presentation of exogenous factor peptides by major histocompatibility class (MHC) II and the regulation of antibody development by various immune-response molecules.[8] A systematic review and meta-analysis investigating the effect of genetic variants in immune-response-related genes on risk of inhibitor development indicated variants in two MHC II genes, tumor necrosis factor alpha, and interleukin 10 were significantly associated with inhibitor risk.[163] Inclusion of these genetic variants in a genetic-based risk prediction tool could enhance tool performance, as these variants my help explain a proportion of the variance in inhibitor risk not related to the hemophilia genotype. Furthermore, categorization of variants for each of these tools is relatively broad, considering the more than 2,500[71] F8 variants known to cause hemophilia. This investigation was not sufficiently powered to detect small differences in

inhibitor risk introduced by rare variants seen in only one or two participants, thus some sort of categorization was necessary. Perhaps finer categorization could enhance tool performance, as a subset of variants with large impact on risk of inhibitors may currently be collapsed together with variants exhibiting less effect. The My Life, Our Future project has genotyped over 3,000 PWH.[69] The results of this project could enable finer classification of hemophilia-causing variants in a way that would better able risk prediction.

In addition to the power issue discussed above, this investigation is also limited in the ability to describe the generalizability of these tools to other populations. Inhibitors occur more frequently in black and Hispanic PWH.[2] In order to minimize the effect of race/ethnicity on evaluating tool performance, participants of non-white race were excluded from this analysis. It is not clear how the tools presented here would perform in these minority population. We intend to investigate this in future efforts. Furthermore, the utility of these tools for predicting inhibitor risk in patients with no identified hemophilia-causing variant may be limited. Over 2% of all participants in our investigation did not have a hemophilia-causing variant identified, most commonly among mild and moderate PWH. Deep intronic variation and variation in genes other than *F8* that produce a hemophilia-like phenotype can not be ruled out for these participants, as our genotyping methods would not have detected such variation. The appropriate categorization of such variation in a way that would enable inhibitor risk prediction among this sub-population is not clear.

Conclusions

The results of this analysis indicate that an inhibitor risk prediction tool based only on broad categorization of the HA-causing variant will be of limited utility. Perhaps inclusion of other genetic variants related to inhibitor development could increase the utility of a score based only on genetic variation.

Acknowledgements

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Tables

	All			Mild			Moderate			Severe		
	All N (%)	**Inhibitor N (%)**	**No Inhibitor N (%)**	**All N (%)**	**Inhibitor N (%)**	**No Inhibitor N (%)**	**All N (%)**	**Inhibitor N (%)**	**No Inhibitor N (%)**	**All N (%)**	**Inhibitor N (%)**	**No Inhibitor N (%)**
Large Deletion	27 (3.6)	16 (9.9)	11 (1.8)	0 (0)	0 (0)	0 (0)	1 (0.9)	1 (7.1)	0 (0)	23 (4.8)	12 (10.1)	11 (3)
Single Exon	14 (1.8)	7 (4.3)	7 (1.2)	0 (0)	0 (0)	0 (0)	1 (0.9)	1 (7.1)	0 (0)	11 (2.3)	4 (3.4)	7 (1.9)
Multi Exon	13 (1.7)	9 (5.6)	4 (0.7)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	12 (2.5)	8 (6.7)	4 (1.1)
Inversion	232 (30.6)	70 (43.2)	162 (27.2)	1 (0.7)	0 (0)	1 (0.7)	5 (4.5)	0 (0)	5 (5.2)	218 (45.2)	62 (52.1)	156 (43)
Intron 22	224 (29.6)	68 (42)	156 (26.2)	1 (0.7)	0 (0)	1 (0.7)	5 (4.5)	0 (0)	5 (5.2)	210 (43.6)	60 (50.4)	150 (41.3)
Intron 1	8 (1.1)	2 (1.2)	6 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	8 (1.7)	2 (1.7)	6 (1.7)
Frameshift	86 (11.3)	21 (13)	65 (10.9)	0 (0)	0 (0)	0 (0)	5 (4.5)	3 (21.4)	2 (2.1)	78 (16.2)	15 (12.6)	63 (17.4)
Inside Poly-A	52 (6.9)	12 (7.4)	40 (6.7)	0 (0)	0 (0)	0 (0)	3 (2.7)	2 (14.3)	1 (1)	47 (9.8)	8 (6.7)	39 (10.7)
Outside Poly-A	34 (4.5)	9 (5.6)	25 (4.2)	0 (0)	0 (0)	0 (0)	2 (1.8)	1 (7.1)	1 (1)	31 (6.4)	7 (5.9)	24 (6.6)
Missense	292 (38.5)	29 (17.9)	263 (44.1)	136 (90.7)	12 (92.3)	124 (90.5)	82 (74.5)	9 (64.3)	73 (76)	73 (15.1)	7 (5.9)	66 (18.2)
Inside Light Chain	74 (9.8)	5 (3.1)	69 (11.6)	25 (16.7)	1 (7.7)	24 (17.5)	27 (24.5)	2 (14.3)	25 (26)	22 (4.6)	2 (1.7)	20 (5.5)
Outside Light Chain	218 (28.8)	24 (14.8)	194 (32.6)	111 (74)	11 (84.6)	100 (73)	55 (50)	7 (50)	48 (50)	51 (10.6)	5 (4.2)	46 (12.7)
Nonsense	64 (8.4)	16 (9.9)	48 (8.1)	1 (0.7)	0 (0)	1 (0.7)	2 (1.8)	0 (0)	2 (2.1)	60 (12.4)	15 (12.6)	45 (12.4)
Inside Light Chain	11 (1.5)	1 (0.6)	10 (1.7)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	11 (2.3)	1 (0.8)	10 (2.8)
Outside Light Chain	53 (7)	15 (9.3)	38 (6.4)	1 (0.7)	0 (0)	1 (0.7)	2 (1.8)	0 (0)	2 (2.1)	49 (10.2)	14 (11.8)	35 (9.6)
Splice Site	18 (2.4)	2 (1.2)	16 (2.7)	5 (3.3)	0 (0)	5 (3.6)	4 (3.6)	0 (0)	4 (4.2)	9 (1.9)	2 (1.7)	7 (1.9)
Other	20 (2.6)	4 (2.5)	16 (2.7)	4 (2.7)	1 (7.7)	3 (2.2)	4 (3.6)	1 (7.1)	3 (3.1)	12 (2.5)	2 (1.7)	10 (2.8)
Synonymous	3 (0.4)	0 (0)	3 (0.5)	2 (1.3)	0 (0)	2 (1.5)	1 (0.9)	0 (0)	1 (1)	0 (0)	0 (0)	0 (0)
Small Rearrangement	6 (0.8)	0 (0)	6 (1)	1 (0.7)	0 (0)	1 (0.7)	0 (0)	0 (0)	0 (0)	5 (1)	0 (0)	5 (1.4)
Large Rearrangement	7 (0.9)	3 (1.9)	4 (0.7)	1 (0.7)	1 (7.7)	0 (0)	1 (0.9)	1 (7.1)	0 (0)	5 (1)	1 (0.8)	4 (1.1)
Large Duplication	4 (0.5)	1 (0.6)	3 (0.5)	0 (0)	0 (0)	0 (0)	2 (1.8)	0 (0)	2 (2.1)	2 (0.4)	1 (0.8)	1 (0.3)
None	19 (2.5)	4 (2.5)	15 (2.5)	3 (2)	0 (0)	3 (2.2)	7 (6.4)	0 (0)	7 (7.3)	9 (1.9)	4 (3.4)	5 (1.4)

Table 1: Distribution of hemophilia A-causing variant types by disease severity and inhibitor status.

Figures

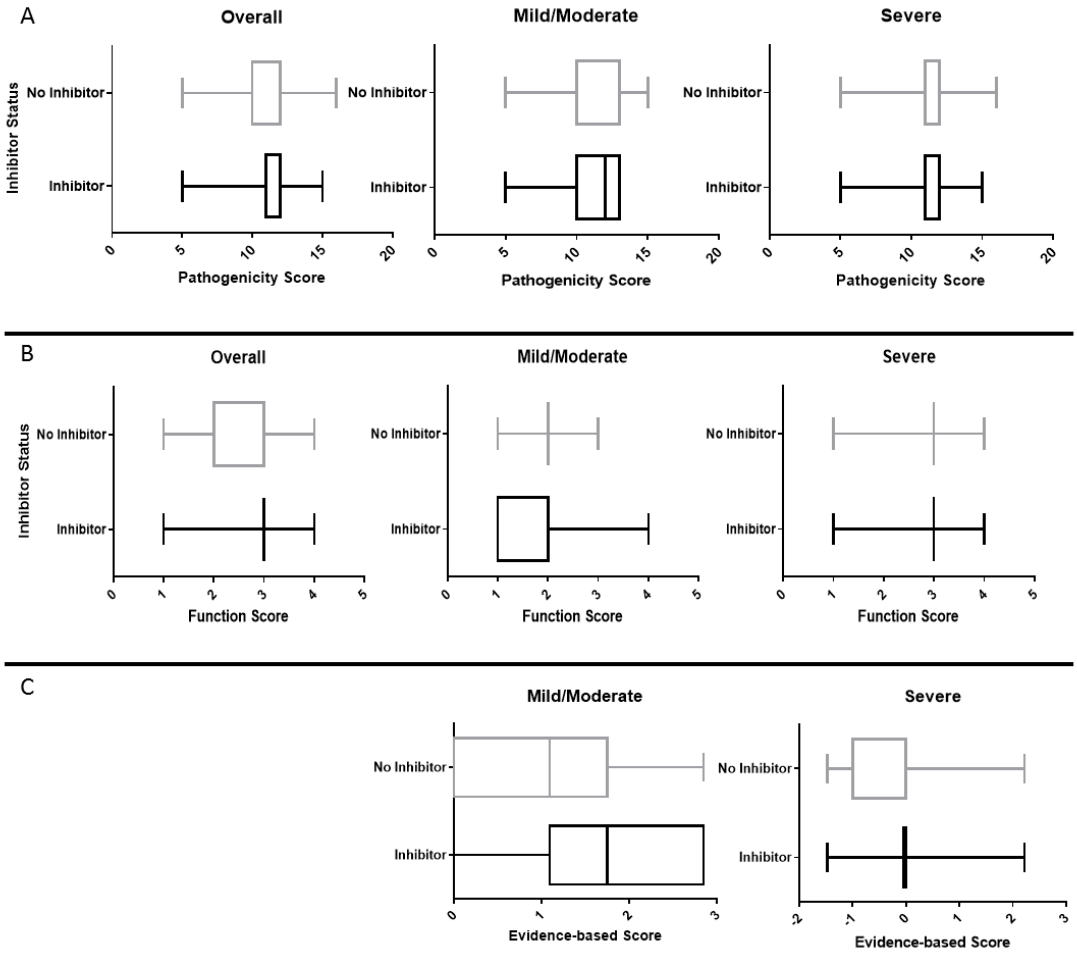

Figure 1: Distribution of prediction scores overall and by severity. A: Pathogenicity score based on American College of Medical Genetics guidelines[133] for predicting pathogenicity of variants. B: Function score assigned based on likelihood variant would lead to production of protein product. C: Evidence-based score assigned in a severity-specific manner using previously-published[10,31] estimates of effect of variant types on risk of inhibitor development.

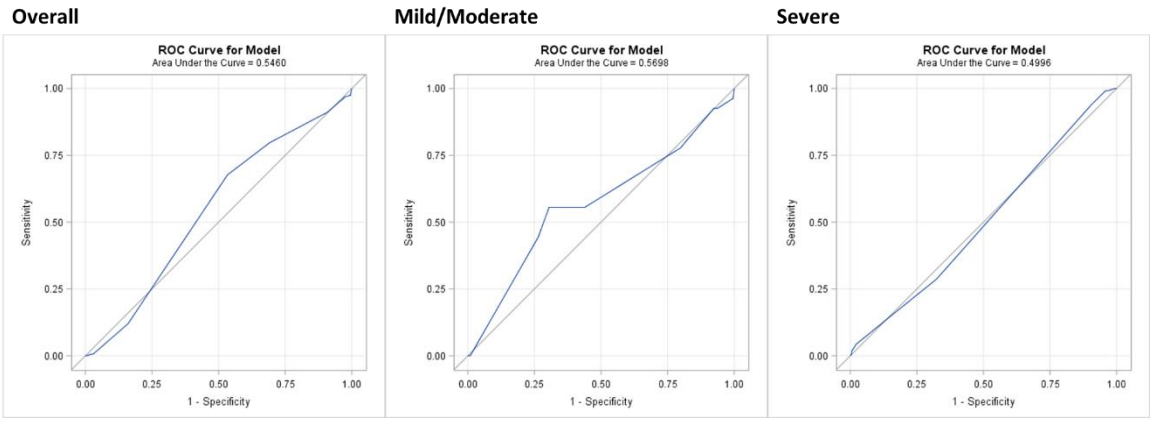

Figure 2: Receiver operating curves and Area Under the Curve statistics for pathogenicity tool, overall and stratified by disease severity.

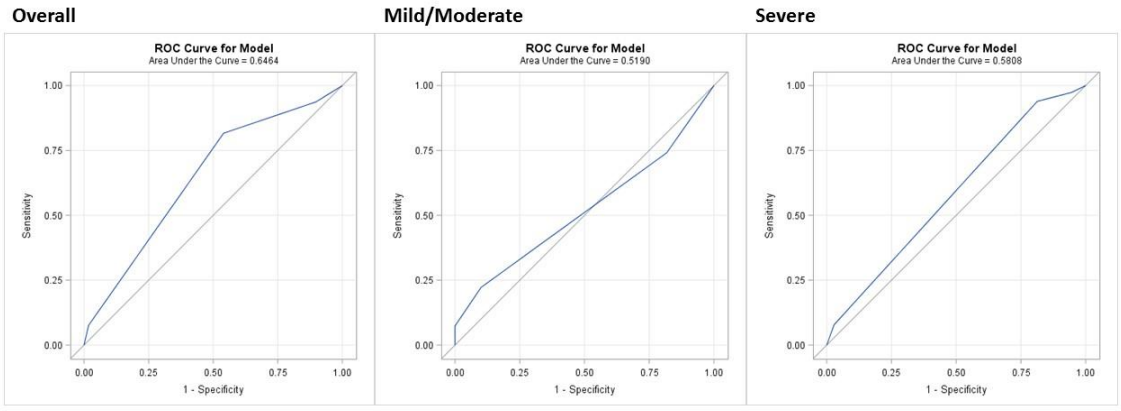

Figure 3: Receiver operating curves and Area Under the Curve statistics for function tool, overall and stratified by disease severity.

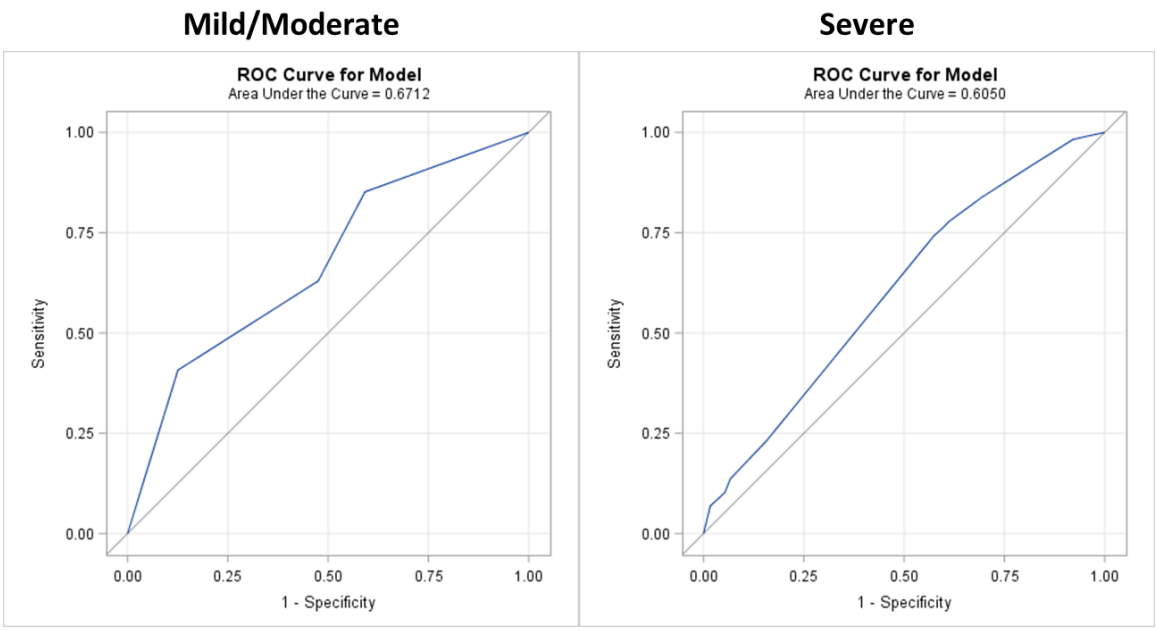

Figure 4: Receiver operating curves and Area Under the Curve statistics for evidence-based tool, overall and stratified by disease severity.

Chapter 4: Genetic variants associated with inhibitors among persons with hemophilia: A systematic review and meta-analysis

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Abstract

Antibodies developed against treatment products, inhibitors, are a serious complication of treatment among persons with hemophilia (PWH). It has been hypothesized that inhibitor development has a genetic component. Because inhibitor development is an immune response, genetic variants in immune-response-related genes could affect inhibitor risk. This systematic review and meta-analysis attempts to identify variants in immune-response-related genes that are consistently associated with inhibitors among PWH. Studies published before August 2016 that were designed to assess the association between variants in immune-response genes and inhibitors among PWH were included. Studies were excluded if they included females or patients with acquired hemophilia. A meta-analysis was conducted for variants reported in at least 3 cohorts. Odds ratios (ORs) were computed to compare the frequency of the genetic variant in PWH with versus without inhibitors in relation to a reference allele. ORs were combined into a summary estimate of effect using a random effects model. Twenty-nine of 629 references indexed in PubMed meeting the search criteria also met the inclusion criteria; 4

additional references were identified by screening references. Summary estimates of effect were calculated for 94 genetic variants in 13 genes. Five variants in 4 genes were significantly associated with inhibitors among PWH. These included two variants in Class II HLA genes (HLA-DQB1*0602: OR 1.77 [95% CI: 1.24-2.53] and HLA-DRB1*15: OR 1.64 [95% CI: 1.18-2.28]) and one variant in TNFA (rs1800629: OR 1.25 [95% CI: 1.01-1.54]). Two variants in IL10 were associated with decreased prevalence of inhibitors (-1082 G>A rs1800896: OR 0.74 [95% CI: 0.57-0.95] and microsatellite repeat rs2234662: OR 0.58 [95% CI: 0.34-1.01]). Numerous studies have investigated the association between variants in immune-response genes and inhibitors among PWH. However, few studies investigated the same sets of genes, and very few variants have been consistently associated with inhibitors. These data suggest the need for systematic, unbiased genome-wide studies to identify genetic predictors of inhibitors.

Introduction

Hemophilia A, an inherited bleeding disorder caused by mutations in the gene that codes for the pro-coagulant protein factor VIII (FVIII), affects approximately 20,000 males in the United States.[1] Hemophilia A is most often treated by replacing the missing factor using either recombinant or plasma-derived FVIII concentrates; however, treatment is not without risk. Approximately 1 in 5 persons with hemophilia (PWH) will develop antibodies to treatment products, known as inhibitors.[2] Inhibitor development has been associated with increased risk for bleeding[3] and early mortality[4], increased factor product utilization[3], and increased healthcare costs[39].

Some efforts have been undertaken to develop risk stratification tools to better predict which PWH will develop inhibitors.[7,164] For example, the risk stratification tool[7] developed by the CANAL study group includes three components: family history of inhibitor development, the patient's *F8* mutation, and intensity of first factor exposure. Some have argued, however, that a major drawback to this tool is its reliance on product exposure and family information and that a tool based solely on genetic markers could be more useful in a clinical setting.[8]

Antibody development to factor concentrates is believed to be a T-cell-mediated process.[8] Antigen-presenting cells endocytose and process the foreign factor protein and present fragments to naïve T helper cells. Naïve T helper cells become activated via a series of immune response signals involving cytokine release and interaction with co-stimulatory molecules. Activated T helper cells interact with antibody-producing B cells to produce antibodies against the foreign factor product.

Because a complex series of interactions with various immune response molecules underlies inhibitor development, genetic variation in these genes could underlie variability in inhibitor development risk. Indeed, several investigations have examined the association between genetic variants in immune response genes and inhibitor development among PWH.[59,76,77,79-87,90,92,93,95-97,99-112,165] However, the degree of consistency in methods and results among the various investigations is unclear. The goal of this systematic review and meta-analysis is to summarize the evidence regarding the associations between genetic variants in immune response genes and inhibitor development among PWH and to evaluate the strength and consistency of the associations that have been investigated. With these data, the feasibility of developing a risk prediction tool based solely on genetic markers can be better understood.

Methods

A literature search was conducted on August 19, 2016, by ABP, a biologist in the Division of Blood Disorders, Centers for Disease Control and Prevention, to identify peer-reviewed publications written in English on genetic association studies of immune response genes and inhibitors among PWH. PubMed was searched using the following terms: (Hemophilia AND Inhibitor AND (Gene* OR MHC OR Major Histocompatibility Complex OR HLA OR Human Leukocyte Antigen OR allele OR polymorphism)). Web of Science was subsequently used to identify records that were cited by or that cited the reports identified via the PubMed search. The resulting list of publications was examined using EndNote Version 7 to identify observational studies (prospective and retrospective cohort, case-control, and cross-sectional

studies) that investigated genetic variants in immune response genes and inhibitors to FVIII among males with hereditary hemophilia A. Publication review was done in a stepwise manner: first examining the titles and abstracts to identify publications not meeting the inclusion criteria and subsequently examining the full text of each manuscript passing the review of title and abstract.

Studies meeting the criteria described above were included in the systematic review. Genetic variants assessed in at least 3 unique cohorts were also included in a meta-analysis of the association of those variants with inhibitor development. Data were extracted from each full-text publication by ABP using a standardized data extraction form (Supplemental Material).

To estimate the effect of each genetic variant included in the meta-analysis on prevalence of inhibitors, odds ratios (ORs) were calculated from summary statistics reported in the original publication, by comparing the proportions of inhibitor-positive and inhibitor-negative persons homozygous for the reference allele to the proportions of inhibitor-positive and inhibitor-negative persons carrying at least one variant allele as reported in each publication. The reference allele was defined based on Genome Reference Consortium Human Build 38 patch release 7. For human leukocyte antigen (HLA) reference alleles not seen in a cohort, a fixed count of one inhibitor patient and one non-inhibitor patients was used.[166] For studies where results were not reported in a manner that would allow the computation of the OR and associated variance, the corresponding author was contacted on two occasions by separate emails sent two weeks apart. If no response was received after four weeks from the date the final email was sent, the data were excluded from analysis. Summary estimates of effect were calculated using a random effects model[167] via the Mantel-Haenszel method[168].

The quality of each study included in the systematic review and/or meta-analysis was assessed using the HuGENet guidelines for assessing the quality of genetic association studies.[169] These guidelines outline ways to assess the quality of genetic association studies based on evaluation

of bias in phenotype definition, bias in genotyping, the potential for population stratification, and bias induced by selective reporting of results. Heterogeneity across studies was assessed for each variant investigated by evaluating the Q statistic (a standardized measure of the deviation of each study from the summary effect estimate), τ^2 (an estimate of the variance of the true effect sizes), and I^2 (the proportion of observed variance that reflects real differences in effect sizes).[170] Publication bias was assessed by examining asymmetry in funnel plots constructed for each variant investigated. Any asymmetry in the funnel plot was noted as suggestion of publication bias.[171]

All calculations and plots were conducted using Review Manager version 5.3.[172]

Results

Study identification and selection

Search of PubMed yielded 629 publications. Of these, 211 were excluded based on the title and a further 378 were excluded based on information provided in the abstract. The full text of the remaining 40 publications was reviewed, which resulted in 11 additional exclusions. Finally, an additional 4 relevant publications were identified via Web of Science by assessing citations either cited by or that cited the remaining 29 manuscripts. Figure 1 outlines the reasons for publication exclusion at each step. The majority of manuscripts excluded in the review of manuscript title were excluded because the investigation was not done in a cohort of persons with hemophilia A, and the majority of manuscripts excluded in the review of manuscript abstract and full text were excluded because the investigation did not report effects of immune response variants.

Study characteristics

The characteristics of each study included in the systematic review and meta-analysis are detailed in Table 1. Most studies were cross-sectional and included severe (FVIII:C <1%) patients only. The largest study (Lozier et al 2011[79]) was a multi-center study that investigated the effect of various immune response related variants on the prevalence of inhibitors using a

tag-SNP approach. The smallest study (Lippert et al 1990[112]) was a single-center study that investigated the effect of HLA type on prevalence of inhibitors. The effect of immune response-related genetic variants on the prevalence of inhibitors was investigated among a wide variety of racial and ethnic groups. Several reports (e.g. Astermark et al 2006a[99], Astermark et al 2006b[100], and Astermark et al 2007[101]) included the results of multiple genetic variants evaluated in the same cohort.

Variants investigated

Among all papers included in this review, the associations between 14,465 unique variants and inhibitor status among PWH were reported (see Supplementary Material for complete list of variants). However, most of these variants were investigated in one cohort only, eliminating the possibility of comparisons across studies. The strongest effect was reported in *DOCK2* (rs1863993, OR 4.29 [95% CI: 2.09-8.80]) by Astermark et al 2013[102]. Unfortunately, this variant was not investigated in any other study. The most common variants tested across all studies were the *HLA-DQB1* and *HLA-DRB1* alleles, investigated in 11 cohorts.

Summary estimates of effect

There were 94 variants investigated in at least 3 cohorts. Table 2 lists the variants included in the meta-analysis, grouped by gene function and gene. Summary estimates of effect and forest plots for all variants included in the meta-analysis are provided in the Supplementary Material. The main findings of these analyses are presented below under the appropriate gene function category.

T cell regulators

The systematic review of the literature identified 3 variants in 2 genes involved in T cell regulation that were investigated in at least 3 cohorts of PWH. Both variants in CTLA4 (rs5742909 and rs231775) and the variant in PTPN22 (rs2476601) were associated with a decreased prevalence of inhibitors. The summary estimates of effect ranged from OR 0.85

(95% CI: 0.65-1.13) (rs231775) to OR 0.93 (95% CI: 0.48-1.79) (rs2476601). None were statistically significantly associated with inhibitors.

Class I HLA genes

The systematic review of the literature identified three reports that investigated the association between Class I HLA genes and inhibitors among PWH. The estimates of effect varied widely among cohorts for each of the alleles included in the meta-analysis. None of the alleles were statistically significantly associated with inhibitors.

Class II HLA genes

Several groups have investigated the effect of Class II HLA variants on the prevalence of inhibitors among PWH. The most commonly investigated genes were HLA-DQB1 and HLA-DRB1, which were investigated in 12 reports.

Compared to the reference allele, none of the HLA-DQA1 alleles were statistically significantly associated with the prevalence of inhibitors, although HLA-DQA1*0102 was consistently associated with slightly increased prevalence of inhibitors (OR 1.47 [95% CI: 0.80-2.69]) and HLA-DQA1*0103, HLA-DQA1*04, HLA-DQA1*05, HLA-DQA1*0501 were consistently associated with slightly decreased prevalence of inhibitors (OR 0.40 [95% CI: 0.15-1.07], OR 0.71 [95% CI: 0.31-1.65], OR 0.70 [95% CI: 0.46-1.06], and OR 0.79 [95% CI: 0.79-1.49], respectively).

Compared to the reference allele, HLA-DQB1*0602 was statistically significantly associated with increased prevalence of inhibitors among PWH (OR 1.77 [95% CI: 1.24-2.53], see Figure 2a). At 2 digit resolution, HLA-DQB1*06 was also associated with increased prevalence of inhibitors, although not significantly (OR 1.19 [95% CI: 0.95-1.49]). Although not statistically significant, HLA-DQB1*0502 was consistently associated with increased prevalence of inhibitors (OR 1.69 [95% CI: 0.96-2.97]) compared to the reference allele. HLA-DQB1*0402, a relatively rare allele,

was also consistently associated with increased prevalence of inhibitors (OR 1.73 [95% CI: 0.83-3.63]) compared to the reference allele.

Compared to the reference allele, HLA-DRB1*15 was statistically significantly associated with increased prevalence of inhibitors among PWH (OR 1.64 [95% CI: 1.18-2.28], see Figure 2b). None of the other HLA-DRB1 alleles were consistently associated with increased or decreased prevalence of inhibitors among PWH.

Cytokines

The systematic review of the literature identified 12 variants in 5 cytokine genes that were investigated in at least 3 cohorts of PWH. None of the variants in *IL1*, *IL4*, or *IL13* were statistically significantly associated with inhibitors. Of the 5 variants investigated in *IL10*, one variant (rs1800896) was significantly associated with decreased prevalence of inhibitors (OR 0.74 [95% CI: 0.57-0.95], see Figure 2c). Another *IL10* variant, a dinucleotide repeat in the promoter region of the gene (rs2234662), was also marginally statistically significantly associated with decreased prevalence of inhibitors (OR 0.58 [95% CI: 0.34-1.01], see Figure 2d). Finally, 1 of the 4 variants investigated in TNFA (rs1800629) was marginally statistically significantly associated with increased prevalence of inhibitors (OR 1.25 [95% CI: 1.01-1.54], see Figure 2e).

Evaluation of heterogeneity

For each variant included in the meta-analysis, measures of heterogeneity, including τ^2, were estimated (see Supplementary Material for all calculations). Overall heterogeneity estimates were low, indicating the confidence intervals often overlapped and only a small amount of the variability observed was due to heterogeneity between studies.

Evaluation of bias

The possible impact of bias, namely publication bias and study-related bias that could contribute to phenotype misclassification, genotype misclassification, and confounding, was evaluated for each variant included in the meta-analysis.

Publication bias

Funnel plots for the variants significantly associated with inhibitors are provided in Figure 3. Due to the relatively small number of studies investigating each variant, the funnel plots were examined and were found to be uninformative. The funnel plot for HLA-DQB1*0602 appears slightly asymmetric, indicating the possible presence of publication bias. Funnel plots for all variants included in the meta-analysis are provided in the Supplementary Material.

Study-related bias

Table 3 outlines various aspects of study methodology that could contribute to bias for each study included in the systematic review. The most common methodologic issues included unclear phenotype definition (definition of inhibitor-positive, inhibitor-negative, or both) and unclear reporting of steps taken to ensure the quality of genotyping data. Population stratification was often not overtly addressed and was, instead, addressed by only including participants from a single ethnic background.

Discussion

Because of its detrimental effect on treatment cost and bleeding risk, inhibitor development among PWH has now become the most concerning complication of the disease. Efforts to understand the characteristics of patients and the environment that increase risk of inhibitor development are important, as this could help identify patients at highest risk for inhibitor development and, perhaps, lead to the development of interventions to minimize risk. Because inhibitor development involves an immune response to exogenous factor product[8], patient characteristics that might influence immune response are important to investigate. Genetic information, in particular, could be deployed to identify PWH who are at risk for inhibitor development *prior* to treatment, allowing for targeted intervention to avoid complications. This systematic review and meta-analysis attempted to summarize and quantify whether genetic variation in immune response genes is associated with inhibitor occurrence among PWH.

The published literature was systematically reviewed for reports of investigations of immune-response-related genetic variants and their association with inhibitors. Authors of studies included in the systematic review and meta-analysis were contacted to attempt to gather all available evidence. For variants investigated in at least 3 cohorts, summary estimates of effect were calculated using random effects models. The review identified 33 reports of investigations of the association between immune-response-related genetic variants and inhibitors, and a total of 14,465 unique variants were investigated. Of these, only 0.6% (n = 94) were investigated in at least 3 samples; thus 99.4% of the genetic data were ineligible for inclusion in the meta-analysis. Of variants included in the meta-analysis, 2 variants in Class II HLA genes (HLA-DQB1*0602 and HLA-DRB1*15) and 3 variants in cytokine genes (*IL10* rs1800896, *IL10* rs2234662, and *TNFA* rs1800629) were found to be to be significantly associated with inhibitors among PWH.

HLA-Class II variants were among the first genetic variations to be investigated in association with inhibitor development[112]. The results of this systematic review and meta-analysis indicate that, while most alleles do not appear to be associated with inhibitors, several alleles are consistently, though not statistically significantly, associated with the prevalence of inhibitors among the cohorts investigated. Furthermore, two alleles were shown to be statistically significantly associated with increased prevalence of inhibitors. The HLA-DQB1*0602 allele, found in this meta-analysis to be associated with an increased prevalence of inhibitors among PWH, has been previously associated with a decreased risk of Type 1 diabetes[173], increased risk of multiple sclerosis[174], and increased (though not significant) risk of Guillain-Barre Syndrome[175]. Similarly, the HLA-DRB1*15 allele, also found in this meta-analysis to be associated with increased prevalence of inhibitors, has been previously associated with increased risk of multiple sclerosis[174] and increased risk of red blood cell antibody production[176], among other phenotypes[177]. The lack of association between most of the Class II alleles and inhibitors may be due to the relationship between HLA type and inhibitor development being more complicated than simply carrying a risk or protective allele. A computational study of

non-severe hemophilia A-causing mutations indicated that disease-causing mutation type coupled with the HLA type is important, as some combinations do not induce major histocompatibility complex binding and, thus, would not be expected to induce inhibitor development.[114]Another potential reason for the lack of association in this meta-analysis could be limited power to detect associations, particularly in small studies. The HLA loci are highly polymorphic, with many alleles seen only in a handful of study participants.

The role of cytokines in immune response, particularly response to exogenous material, is important. Cytokines play a role in induction of clonal expansion and differentiation of naïve B cells into antibody-producing plasma cells[178] and in regulation of the immune response[179]. Of the over 200 cytokine genes identified, the association between 133 genes and inhibitors among PWH were investigated in at least one cohort. However, only 12 variants in 5 cytokine genes were investigated in at least 3 cohorts. The systematic review and meta-analysis identified two variants in *IL10* that were associated with decreased prevalence of inhibitors among PWH (rs100896 and rs2234662) and one variant in *TNFA* (rs1800629) that was associated with increased prevalence of inhibitors. *IL10* plays an important role in limiting the immune response[179]. Both variants identified in the systematic review and meta-analysis are in the promoter region of the gene and have been linked to gene expression[180-182]. These variants have also been reported to be associated with a variety of phenotypes[183]. *TNFA* plays an important role in promoting the immune response[184]. The variant identified in this systematic review and meta-analysis as being associated with increased prevalence of inhibitors has been associated with higher *TNF* expression and multiple phenotypes[183].

With the exception of a few multi-national studies[76,79,99-102,108], efforts to examine the effects of genetic variation in immune-response-related genes on risk of inhibitors among PWH have been largely isolated. There has been a general lack of phenotype standardization, genotyping quality control, and control for important confounders such as population stratification. This lack of

standardization limits the conclusions that can be made from this systematic review and meta-analysis. According to the guidelines on the assessment of cumulative evidence of genetic associations[169], unclear phenotype definition and lack of control for population stratification can have a profound impact on the accurate estimation of associations. Another complicating aspect of the proliferation of small, often single-center, studies is the relative lack of statistical power to detect associations. Perhaps evidence of this problematic issue can be noted in the results of this meta-analysis, as several variants indicated consistent directions of effect but were not statistically significant, highlighting the need for a coordinated, multi-national effort to ensure that phenotype definitions are consistent and investigations are sufficiently powered to detect associations. This increase in power would allow more agnostic evaluations, such as whole exome and whole genome investigations, which would not rely on pre-experimental assumptions regarding the genes involved.

Despite the limitations of this systematic review and meta-analysis, this effort remains the largest, most comprehensive review of the evidence regarding the associations between genetic variants involved in immune response and occurrence of inhibitors among PWH. Care was taken to identify all published and unpublished data on this topic and to standardize the data so that summary estimates of effect could be calculated.

Conclusions

The results of this systematic review and meta-analysis indicate that, although many different genetic variants have been investigated, relatively few have been investigated in multiple cohorts. Of those investigated in multiple cohorts, even fewer were statistically significantly associated with inhibitors. Furthermore, there has been a lack of standardized methodology across investigative efforts. This highlights the need for a coordinated approach to investigating the role of immune response genetics in risk of inhibitor development. Based on these results, it currently seems infeasible to produce a risk prediction tool based solely on genetic markers of risk.

Acknowledgements

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

The authors acknowledge Dr. Stephanie L. Sherman for her valuable contribution to the design of this systematic review and meta-analysis and review of the manuscript.

Tables

Reference	Inhibitor + Definition	Inhibitor - Definition	Inhibitor + N	Inhibitor - N	Race/Ethnicity/Nationality	Severity	Study Type	Study Name	Dates Data Collection	Notes
Lippert et al 1990[112]	any titerable Bethesda inhibitor titer	never had a titerable Bethesda inhibitor titer	11	17	NR	MI, MO, S	Cross Sectional		1985-1987	Genotyping by RFLP
Hay et al 1997[110]	any inhibitor titer >10 BU	never had a titerable Bethesda inhibitor titer	52	124	NR	S	Cross Sectional		NR	
Oldenburg et al 1997[83]	repeated inhibitor titer >1 BU	no history of inhibitor in >100 ED	29	42	German	S	Cross Sectional		NR	All Intron 22 Inversion
Ohta et al 1999[82]	NR	NR	20	26	Japanese	NR	Cross Sectional		NR	
Tizzano et al 2002[95]	NR	NR			Spanish	MI, MO, S	Cross Sectional		NR	Contacted author regarding data
Bril et al 2004[105]	NR	NR	7	38	Caucasian	NR	Cross Sectional		NR	All Arg593Cys
Astermark et al 2006a[99]	NR	no history of inhibitor in >100 ED	77	87	Caucasian (N=160) Non-Caucasian (N=4)	MI, MO, S	Cross Sectional	MIBS	NR	
Astermark et al 2006b[100]	NR	no history of inhibitor in >100 ED	77	87	Caucasian (N=160) Non-Caucasian (N=4)	MI, MO, S	Cross Sectional	MIBS	NR	
Astermark et al 2007[101]	NR	no history of inhibitor in >100 ED	63	61	NR	S	Cross Sectional	MIBS	NR	
Kurnik et al 2007[165]	NR	NR	28	94	Caucasian	S	Prospective Cohort		NR	Contacted author regarding data
Wieland et al 2008[77]	NR	NR	25	25	Caucasian Russian African Arabian	NR	Cross Sectional		NR	Contacted author regarding data
Pavlova et al 2009[84]	NR	no history of inhibitor in >150 ED	130	130	German	S	Matched Case-Control		NR	Matched on mutation type Excluded non-null mutations
Ragni et al 2009[59]	inhibitor titer above normal range	NR	65	119	Caucasian African-American	MI, MO, S	Matched Case-Control	Hemophilia Inhibitor Study	NR	Matched on age Contacted author regarding data
Bafunno et al 2010[103]	NR	NR	115	328	Italian	NR	Cross-Sectional	AICE	2000-2006	
Chaves et al 2010[106]	NR	NR	30	30	Brazilian	NR	Case-Control	Fundacao Hemominas	NR	Contacted author regarding data
Lozier et al 2011[79]	any inhibitor titer >1 BU	NR	302	633	Non-Hispanic Caucasian	S	Cross Sectional	Multicenter Hemophilia Cohort Studies I/II	1982-2005	TAG SNP approach Contacted author regarding data See Supplmentary Material for full list of variants tested
Agostini et al 2012[96]	any inhibitor titer >1 BU	NR	39	97	European	S	Cross Sectional		NR	
Bafunno et al 2012[104]	NR	NR	111	97	NR	NR	Cross Sectional	AICE	2000-2006	
De Barros et al 2012[107]	NR	NR	40	131	Caucasian African-American Mulatto	MI, MO, S	Cross Sectional	DATAUSweb	2007-2009	
Lu et al 2012[80]	any Nijmegen-Bethesda inhibitor titer >0.6 BU	no Nijmegen-Bethesda inhibitor titer >0.6 BU	63	59	Chinese	MI, MO, S	Cross Sectional		NR	Only repeats >5% MAF reported for IL10.
Nathalang et al 2012[81]	any Bethesda inhibitor titer >0.6 BU	no Bethesda inhibitor titer >0.6 BU	31	26	Thai	NR	Cross Sectional		NR	

Pinto et al 2012[86]	any Nijmegen-Bethesda inhibitor titer >1 BU	no Nijmegen-Bethesda inhibitor titer >1 BU in at least 10 ED	50	70	Indian	S	Cross Sectional		NR	
Astermark et al 2013[102]	a current or history of a Bethesda inhibitor titer >1 BU measured at the local laboratory	no current or history of a Bethesda inhibitor titer >1 BU measured at the local laboratory	457	376	Caucasian African-American Hispanic Asian Other	MI, MO, S	Cross Sectional	Hemophilia Inhibitor Genetics Study HGDS HIGS MIBS	HIGS: 2004-2010 MIBS: 1996-2000 HGDS: 1989-1990	Genotyped over 14,000 variants in 3 studies. Performed meta-analysis to determine variants significantly associated with inhibitor development.
Pergantou et al 2013[85]	any Bethesda inhibitor titer >0.6 BU	no Bethesda inhibitor titer >0.6 BU in at least 150 ED	28	24	Greek	S	Cross Sectional		1998-2011	Contacted author regarding data
Repesse et al 2013[87]	NR	no history of inhibitor in 150 ED	99	263	French	S	Cross Sectional		NR	
Schwarz et al 2013[76]	a current or history of a Bethesda inhibitor titer >1 BU measured at the local laboratory	no current or history of a Bethesda inhibitor titer >1 BU measured at the local laboratory	52	213	Caucasian African-American Hispanic Other	MI, MO, S	Cross Sectional	Hemophilia Inhibitor Genetics Study HGDS	1989-1990	Contacted author regarding data
Schwarz et al 2013[76]	a current or history of a Bethesda inhibitor titer >1 BU measured at the local laboratory	no current or history of a Bethesda inhibitor titer >1 BU measured at the local laboratory	360	88	Caucasian African-American Hispanic Asian Other	S	Cross Sectional	Hemophilia Inhibitor Genetics Study HIGS	2004-2010	Contacted author regarding data
Eckhardt et al 2014[108]	at least 2 inhibitor titers >= 1 BU	NR	36	49	Caucasian	S	Cross Sectional	MIBS	NR	
Fidanci et al 2014[109]	NR	NR	42	61	Turkish	MO, S	Cross Sectional		NR	All null F8 mutations
Kenet et al 2014[111]	at least 2 locally-defined positive inhibitor titers >5 BU	NR	54	162	Caucasian	MO, S	Cross Sectional		1980-2011	Only included high-titer inhibitor-positive patients
Pinto et al 2014[93]	any Nijmegen-Bethesda inhibitor titer >1 BU	no positive Nijmegen-modified inhibitor titer in >10 years, >10 EDs	56	63	Indian	S	Cross Sectional		NR	Contacted author regarding data
De Alencar et al 2015[97]	NR	NR	35	82	Brazilian	S	Cross Sectional		2007-2009	Contacted author regarding data
Gorski et al 2016[90]	at least 2 Bethesda inhibitor titers >0.5 BU	NR	17	9	Italian	S	Cross Sectional	Discovery	1960-2010	All Intron 22 Inversion Whole Exome Sequencing Contacted author regarding data
Gorski et al 2016[90]	at least 2 Bethesda inhibitor titers >0.5 BU	NR	53	174	Italian	S	Cross Sectional	Replication	1960-2010	All Intron 22 Inversion
Pinto et al 2016[92]	any Nijmegen-Bethesda inhibitor titer >1 BU	no positive Nijmegen-modified inhibitor titer in >10 years, >10 EDs	80	65	Indian	S	Cross Sectional		2012-2014	Contacted author regarding data Extension of results published in 2012 and 2014

Table 1: Characteristics of studies included in systematic review of the association between genetic variants involved in immune response and inhibitors among persons with hemophilia A.

MI=Mild, MO=Moderate, S=Severe, NR=Not Reported, BU=Bethesda Unit

Gene Function	Gene	Variants
T cell regulators	CTLA4	-318 C>T (rs5742909)
		49 A>G (rs231775)
	PTPN22	c.1858 C>T (rs2476601)

Table 2a: Variants in genes involved in T cell regulation included in the meta-analysis

Gene Function	Gene	Variants
Class I HLA genes	HLA-A	A*01 (reference allele)
		A*02
		A*03
		A*11
		A*24
		A*29
		A*30
		A*31
		A*32
		A*33
		A*68
	HLA-B	B*07 (reference allele)
		B*08
		B*13
		B*14
		B*15
		B*35
		B*44
		B*57
	HLA-C	C*01 (reference allele)
		C*02
		C*03
		C*04
		C*05
		C*07

Table 2b: Variants in Class I HLA genes included in the meta-analysis

Gene Function	Gene	Variants	
Class II HLA genes	HLA-DQA1	DQA1*01 (2 digit reference allele)	DQA1*03
		DQA1*0101 (4 digit reference allele)	DQA1*04
		DQA1*0102	DQA1*05
		DQA1*0103	DQA1*0501
		DQA1*02	DQA1*06
		DQA1*0201	
	HLA-DQB1	DQB1*02	DQB1*0502
		DQB1*0201	DQB1*0503
		DQB1*03	DQB1*0504
		DQB1*0301	DQB1*06
		DQB1*0302	DQB1*0601
		DQB1*0303	DQB1*0602
		DQB1*04	DQB1*0603
		DQB1*0402	DQB1*0604
		DQB1*05 (2 digit reference allele)	DQB1*0605
		DQB1*0501 (4 digit reference allele)	DQB1*0609
	HLA-DRB1	DRB1*01 (2 digit reference allele)	DRB1*09
		DRB1*0101 (4 digit reference allele)	DRB1*0901
		DRB1*03	DRB1*10
		DRB1*0301	DRB1*1001
		DRB1*04	DRB1*11
		DRB1*0401	DRB1*12
		DRB1*0402	DRB1*13
		DRB1*07	DRB1*14
		DRB1*0701	DRB1*15
		DRB1*08	DRB1*1501
		DRB1*0801	DRB1*16
		DRB1*0802	

Table 2c: Variants in Class II HLA genes included in the meta-analysis

Gene Function	Gene	Variants
Cytokines	IL1	IL1B TaqI RFLP (rs1143634)
	IL4	-590 C>T (rs2243250)
	IL10	microsatellite (rs2234662)
		-1082 G>A (rs1800896)
		-819 C>T (rs1800871)
		-592 C>A (rs1800872)
		rs3024496
	IL13	2044 A>G (rs20541)
	TNFA	-308 G>A (rs1800629)
		-827 C>T (rs1799724)
		-238 G>A (rs361525)
		670 A>G (rs3093662)

Table 2d: Variants in cytokine genes included in the meta-analysis

		Bias Assessment			
Reference	Study Name	Phenotype	Genotype	Population Stratification	Reporting
Lippert et al 1990[112]		unclear	NR	NR	only reported significant results
Hay et al 1997[110]		clear	NR	NR	reported all results
Oldenburg et al 1997[83]		clear	NR	same descent	did not test uncommon alleles
Ohta et al 1999[82]		unclear	NR	same descent	did not test uncommon alleles
Tizzano et al 2002[95]		unclear	NR	same descent	did not report inhibitor as outcome*
Bril et al 2004[105]		unclear	NR	same descent	reported all results
Astermark et al 2006a[99]	MIBS	unclear	NR (HLA) appropriate QC (TNFA)	NR	reported all results
Astermark et al 2006b[100]	MIBS	unclear	NR	NR	reported all results
Astermark et al 2007[101]	MIBS	unclear	NR	NR	reported all results
Kurnik et al 2008[165]		unclear	NR	same descent	did not report alleles independently*
Wieland et al 2008[77]		unclear	NR	NR	only reported significant results*
Pavlova et al 2009[84]		unclear	appropriate QC	same descent	reported all results
Ragni et al 2009[59]	Hemophilia Inhibitor Study	unclear	NR	matched case/control	did not report alleles independently**
Bafunno et al 2010[103]	AICE	unclear	appropriate QC	same descent	reported all results
Chaves et al 2010[106]	Fundacao Hemominas	unclear	NR	same descent	only reported significant results*
Lozier et al 2011[79]	Multicenter Hemophilia Cohort Studies I/II	unclear	appropriate QC**	same descent	reported all results**
Agostini et al 2012[96]		unclear	appropriate QC	same descent	reported all results
Bafunno et al 2012[104]	AICE	unclear	appropriate QC	NR	reported all results
De Barros et al 2012[107]	DATAUSweb	unclear	NR	NR	reported all results
Lu et al 2012[80]		clear	NR	same descent	reported all results
Nathalang et al 2012[81]		clear	NR	same descent	reported all results
Pinto et al 2012[86]		clear	NR	same descent	reported all results
Astermark et al 2013[102]	Hemophilia Inhibitor Genetics Study HGDS HIGS MIBS	clear	appropriate QC	PCA	only reported significant results*
Pergantou et al 2013[85]		clear	NR	same descent	only reported significant results*
Repesse et al 2013[87]		unclear	appropriate QC	same descent	reported all results
Schwarz et al 2013[76]	Hemophilia Inhibitor Genetics Study HGDS	clear	NR	NR	reported all results**
Schwarz et al 2013[76]	Hemophilia Inhibitor Genetics Study HIGS	clear	NR	NR	reported all results**
Eckhardt et al 2014[108]	MIBS	unclear	NR	same descent	reported all results
Fidanci et al 2014[109]		unclear	appropriate QC	same descent	reported all results
Kenet et al 2014[111]		unclear	NR	modeling adjustment	reported all results
Pinto et al 2014[93]		clear	NR	same descent	reported all results**
De Alencar et al 2015[97]		unclear	NR	same descent	reported all results**
Gorski et al 2016[90]	Discovery	unclear	appropriate QC	same descent	reported all results**
Gorski et al 2016[90]	Replication	unclear	appropriate QC	same descent	reported all results
Pinto et al 2016[92]		clear	NR	same descent	reported all results**

Table 3: Assessment of potential study-related biases.

NR=Not Reported, *contacted author to obtain results (no reply), **contacted author to obtain results (author provided additional information)

Figures

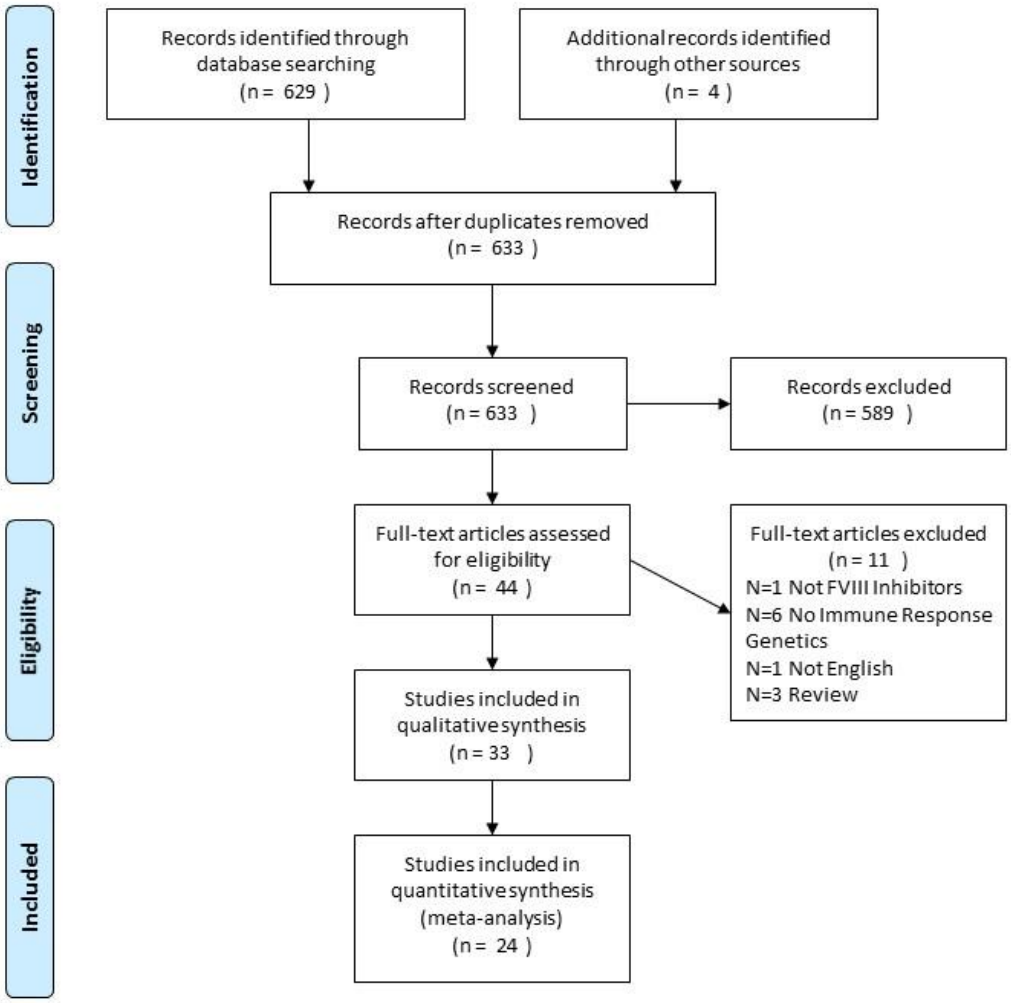

Figure 1: Flow diagram of systematic review of literature to identify investigations assessing the association between genetic variants related to immune response and inhibitors among persons with hemophilia A.

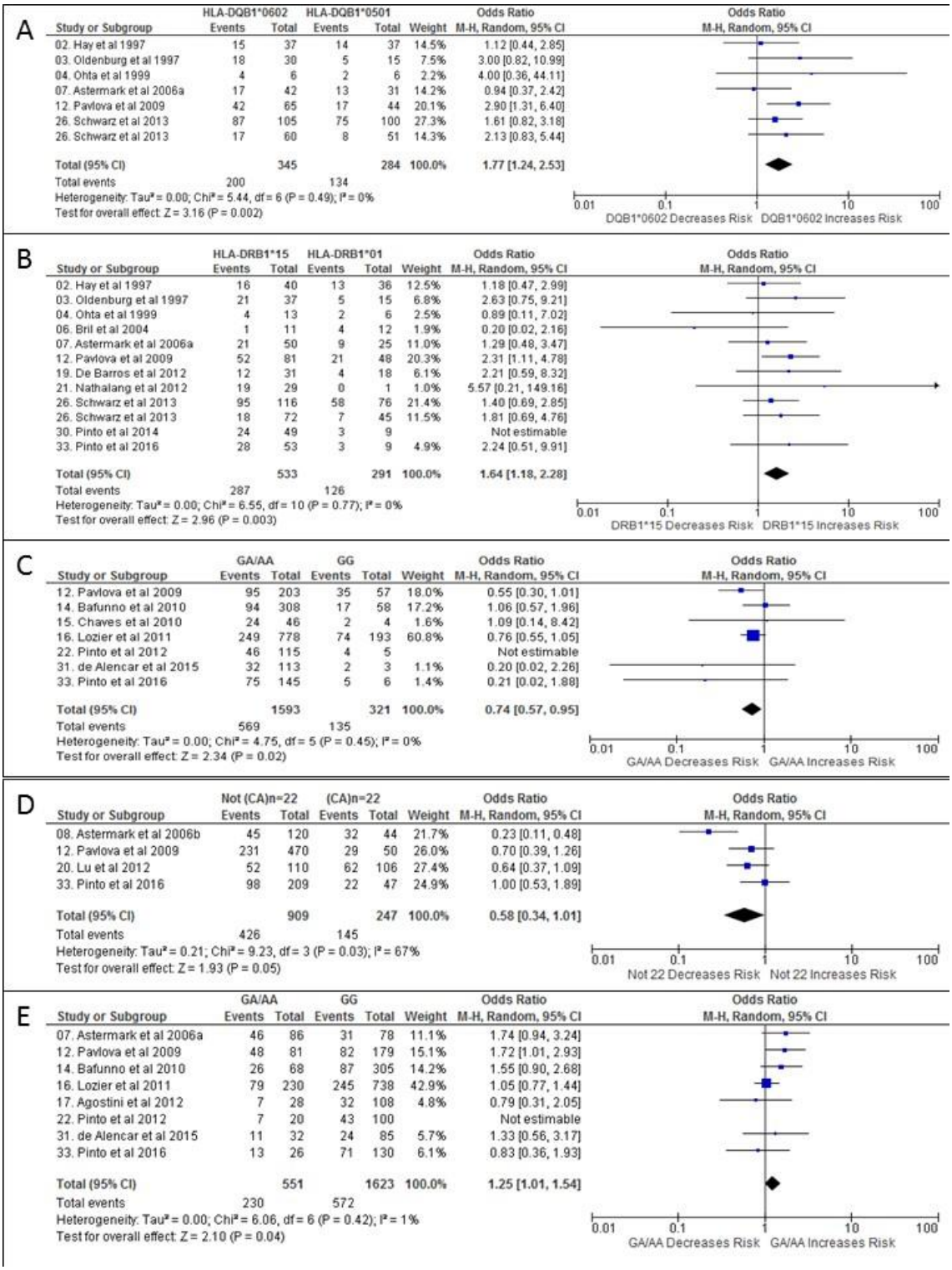

Figure 2: Results of meta-analysis for genetic variants related to immune response significantly associated with inhibitors among persons with hemophilia A.

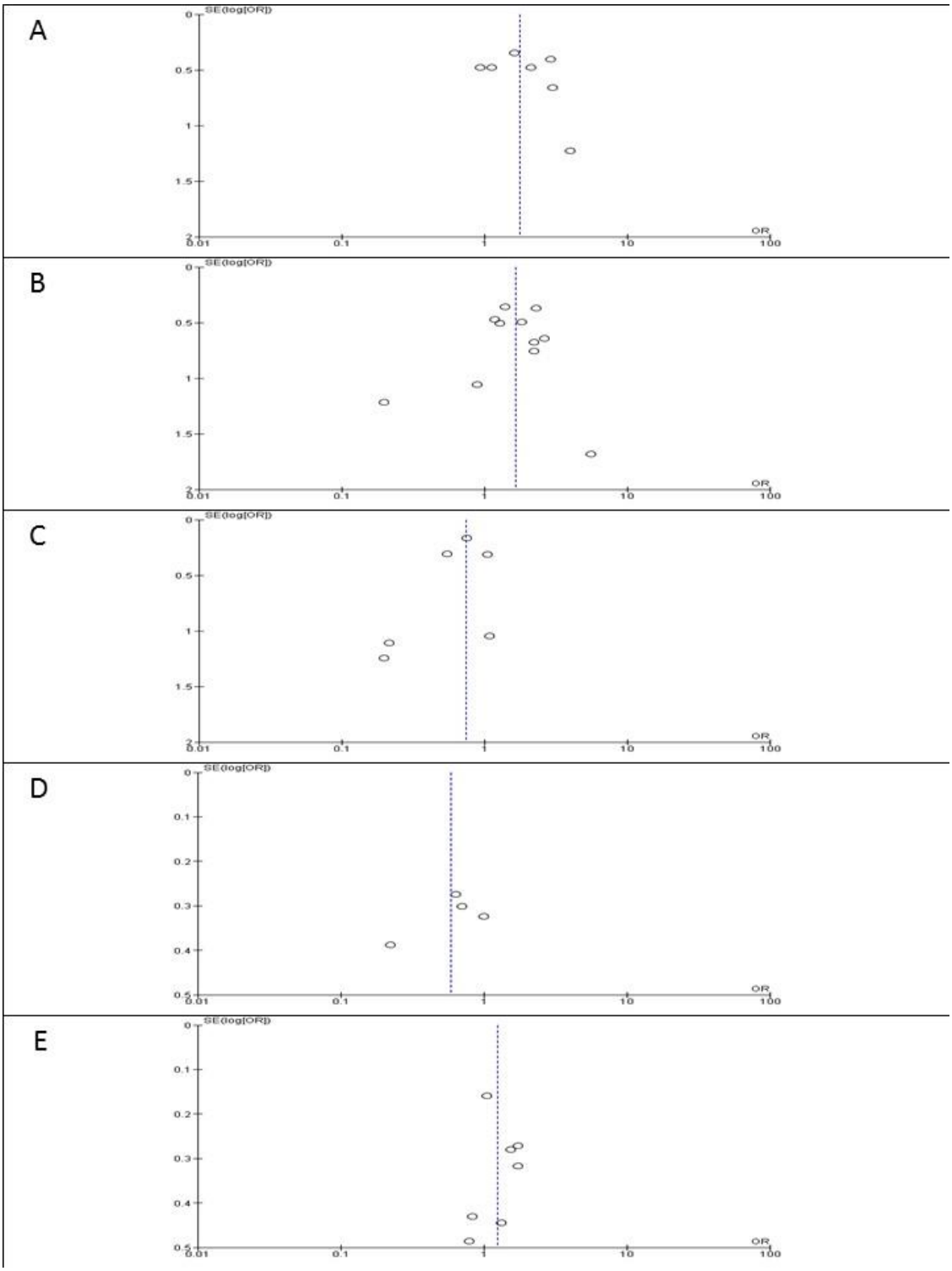

Figure 3: Funnel plots to assess publication bias for genetic variants related to immune response significantly associated with inhibitors among persons with hemophilia A.

Chapter 5: Associations between variants in immune response genes and inhibitors among persons with hemophilia A

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Abstract

Inhibitors, antibodies developed against treatment products rendering them ineffective, affect up to 15% of all persons with hemophilia A (PWH). It has been suggested that there are environmental and genetic components underlying inhibitor risk, with genetic risk factors being both the hemophilia genotype and other variants outside the *F8* locus that may play an important role in immune response modulation. Understanding which genetic variants may be markers of inhibitor risk could help better determine which PWH may be most likely to develop inhibitors. We investigated the association between variants in immune-response-related genes and inhibitors among a large group of PWH. White, Non-Hispanic PWH with and without a history of inhibitors enrolled in the Hemophilia Inhibitor Research Study (HIRS) who consented were genotyped using a 1,536-variant panel that mapped the major histocompatibility complex (MHC) region as well as other immune-response-related genes outside the MHC region. Results from the panel were used to impute MHC Class I and II genotypes. Additionally, dinucleotide repeat polymorphisms which have been demonstrated to affect gene expression were genotyped for two genes (*HMOX1* rs3074372 and *IL10* rs2234662).

Associations between genotypes and inhibitor prevalence were assessed using logistic regression, adjusting for multiple comparisons. Among 758 subjects included in this investigation, variants in *HLA-B*, *HLA-DPB1*, *IL1A*, *IL12B*, *CD80*, and *IL10* were associated with inhibitor prevalence. This investigation highlights the importance of variation in the MHC region in determining inhibitor risk and identifies other genes that may be significant. Many of the variants investigated were selected because of their amenability to mapping rather than ability to influence gene function warranting further investigation into how genetic variation in these genes can influence inhibitor risk. Furthermore, while this investigation included a large group of PWH, the power to detect statistically significant differences between inhibitor-positive and inhibitor-negative enrollees was limited due to the large number of variants investigated, highlighting the need to employ other methodologies to investigate associations among this limited patient population.

Introduction

Hemophilia A, an inherited bleeding disorder currently affecting approximately 20,000 males in the United States[1], is often treated by replacing the missing or dysfunctional factor VIII (FVIII) protein with either plasma-derived or recombinant FVIII[185]. Unfortunately, 10%-15% of persons with hemophilia A (PWH) will develop antibodies to these treatment products, rendering them ineffective.[2] Inhibitors have been associated with increased risk of early death[4], product utilization[3], and healthcare costs[3].

Inhibitor development is a process that involves the presentation of a foreign peptide antigen (therapeutic FVIII) by major histocompatibility complex (MHC) molecules on antigen-presenting cells to antigen-specific T-cells that, with co-stimulatory molecules, promotes proliferation of helper T cells.[8] Proliferation of helper T cells can lead to proliferation of B cells that undergo affinity maturation to become antibody-producing plasma cells.[51]

Risk of inhibitor development has been proposed to have three main components, each altering the likelihood of foreign peptide recognition and stimulation of the immune system: treatment characteristics, patient-related environmental characteristics, and patient genetics.[8] Perhaps the most well-studied inhibitor risk factor is the hemophilia-causing genotype, with variants causing complete loss of gene product being associated with the highest risk of inhibitor development likely because any therapeutic FVIII is seen as foreign by the patient's immune system.[10,31] However, not all patients with a variant that causes complete loss of gene product will develop an inhibitor, highlighting the importance of other components of inhibitor risk, including other genetic components.

The Malmo International Brother Study showed that inhibitor status was highly, though not completely, concordant between family members and indicated a genetic predisposition to inhibitor development outside of shared hemophilia-causing genotype.[53] Several groups have investigated the relationship between genetic variants in immune-response-related genes and hemophilia.[186] Most have relied on genotyping one or two variants in each gene, most of which do not fully map the gene region and are not predicted to alter gene function. We report the results of our investigation of the relationship between variants in immune-response-related genes and inhibitors among persons with hemophilia A by mapping genes involved in immune response and by genotyping variants known to impact gene function.

Methods

Population

Data from males with hemophilia A enrolled in the Hemophilia Inhibitor Research Study (HIRS) were used for this analysis. HIRS has been previously described.[130] Briefly, persons with hemophilia A or hemophilia B were enrolled from 17 participating federally-funded hemophilia treatment centers, regardless of age or hemophilia severity. Standardized data collection tools were used to collect information such as demographic characteristics, previous history of inhibitor, and baseline factor activity level. In order to minimize the effect of

population stratification, only enrollees of White, non-Hispanic race/ethnicity were included in this investigation. If relatives were co-enrolled, data from the first enrolled relative was used for this investigation.

Laboratory Methods

The hemophilia genotype was determined by sequencing the 5' and 3' untranslated regions, all exons, and intron-exon junction regions of *F8* in forward and reverse directions using an automated analyzer (3730 DNA Analyzer, Applied Biosystems, Carslbad, CA, USA) and the Variant SEQr™ protocol. Sequences were analyzed using SeqScape® (Applied Biosystems). Inversion status was determined by PCR.[67,68] For enrollees with no hemophilia-causing variant identified by sequencing or inversion testing, Multiplex Ligation-dependent Probe Amplification was performed (P178-A1 Factor VIII, MRC Holland, Amsterdam, The Netherlands) to detect possible large duplications within *F8*.

A custom 1,536-variant genotyping panel (GoldenGate®, Illumina, San Diego, CA, USA) was designed to map the MHC region on chromosome 6 as well as 27 other genes in the immune response pathway. Variants in the MHC region were chosen based on their ability to fine-map human leukocyte antigen (HLA) genes, as indicated in the design of the Immunochip (Illumina).[187] Variants outside the MHC region were chosen based on their ability to map genes of interest by tagging haplotype blocks as well as candidate variants previously reported to be associated with inhibitors. Haplotype blocks were identified by examining the linkage disequilibrium between variants reported in the HapMap CEU and YRI populations using the Genome Variation Server (http://gvs.gs.washington.edu/GS150) available through SeattleSNPs (http://pga.gs.washington.edu). Variants showing adequate separation upon visual inspection of the scatter plots were included in this analysis.

HMOX1 promoter $(GT)_n$ dinucleotide repeat (rs3074372) length was determined using PCR fragment size analysis as previously described.[188,189] *HMOX1* $(GT)_n$ repeat sizes were grouped as

small (S) (≤ 25 repeats) and large (L) (> 25 repeats). *IL10* microsatellite $(CA)_n$ repeat (rs2234662) length was determined by a similar method, using a FAM labeled forward primer, GT CCT TCC CCA GGT AGA GCA ACA CTC C and an unlabeled reverse primer, CTC CCA AAG AAG CCT TAG TAG TGT TG. Amplification was performed in a 10 μl reaction (2.15 ul water, 5.00 ul Amplitaq Gold 360 [Applied Biosystems], 1.6 ul 50% glycerol, and 0.125 ul each primer at 50 uM). The PCR conditions used for labeling were 96°C for 10 minutes, 29 cycles (94°C for 30 seconds; 62°C for 30 seconds; 72°C for 30 seconds) and 72°C for 7 minutes, followed by a 4°C hold. Labeled products were run with an internal size standard (GeneScan™ –500 LIZ®, Applied Biosystems) in Hi-Di™ Formamide (Applied Biosystems) on a 3730 DNA Analyzer, and fragment size was determined using GeneMapper® Software Version 4.0 software (Applied Biosystems). *IL10* $(CA)_n$ repeat sizes were grouped as small (S) (≤ 18 repeats) and large (L) (> 18 repeats).

Statistical Analysis

Data on the genotyping panel were split based on whether or not the variant was in the MHC region. Data on variants in the MHC region were used to impute HLA genotypes for *HLA-A*, *HLA-B*, *HLA-C*, *HLA-DPB1*, *HLA-DQA1*, *HLA-DQB1*, and *HLA-DRB1* via HIBAG to 2-digit resolution.[190] Data from variants outside the MHC region were investigated using PLINK.[191] Individuals missing greater than 10% of data for these variants were excluded. Variants were excluded based on amount of genotyping failure (>5%), Hardy-Weinberg equilibrium failure ($p<0.001$), and minor allele frequency (<5%). The variant list was pruned based on linkage disequilibrium ($R^2=0.5$) to increase the power to detect an association. Top candidate variants were identified by generating significance levels empirically using permutation procedures. Variants with an empiric p value <0.05 were considered top candidates. The association between genotypes and prevalence of inhibitors was assessed using logistic regression (SAS

version 9.4, SAS Institute, Cary, North Carolina, USA) using a dominant model. Confidence intervals for odds ratios were adjusted using the Benjamini-Hochberg method to account for multiple comparisons.[192]

Results

Among 1,300 enrollees in HIRS, 758 were eligible for this investigation. Characteristics of enrollees are outlined in Table 1. Twenty-one percent either had a history of inhibitor development or were inhibitor positive at the time of the study. The majority of enrollees had severe disease and had greater than 150 exposure days to factor concentrates at the time of enrollment. *F8* inversions were the most common variants among both inhibitor-positive and inhibitor-negative enrollees, followed by missense variants.

HLA Class I and II Variants

The HLA genotype of 628 (83%) of 758 eligible enrollees was imputed based on data from a custom genotyping panel designed to map the MHC region and other variants in genes in the immune response pathway. Of the 53 variants in MHC Class I genes identified, only 2 were significantly associated with prevalence of inhibitors in this study (Table 2). The common *HLA-A**03 allele was associated with increased prevalence of inhibitors compared to the reference allele (OR 1.73 [1.00-2.99]). However, this association is likely due to chance since the strength of the association was not consistent across severity categories or among enrollees with a type 1 intron 22 inversion (Supplemental Table 1). On the other hand, the rare *HLA-B**37 allele was associated with increased prevalence of inhibitors compared to the reference allele (OR 4.03 [1.14-14.20]) and the strength of this association was similar across all severity categories (Supplemental Table 1). Of the 34 variants in MHC Class II genes identified, 4 variants were significantly associated with prevalence of inhibitors (Table 3). These four variants were all *HLA-DPB1* variants and were associated with increased prevalence of inhibitors compared to the rare reference allele *HLA-DPB1**01. While most were not statistically significant, the

associations of these four variants with increased prevalence were consistent across severity categories and among enrollees with type 1 intron 22 inversions (Supplementary Table 2).

Other Variants

Among the 331 variants outside the MHC region investigated on a custom genotyping panel that met clustering criteria, 122 were excluded for one or more of the following reasons: failed test of Hardy-Weinberg equilibrium (N=5), high genotyping failure rate (N=10), or low minor allele frequency (N=110). The remaining 209 variants were further pruned based on linkage disequilibrium to produce an independent set of 93 variants to investigate. Top candidate variants identified by empirically generating significance levels using permutation procedures are listed in Table 4. A variant in *IL1A* (rs17561) and a variant in *IL12B* (rs1003199) were associated with decreased prevalence of inhibitors compared to reference alleles (OR 0.58 [0.30-1.14] and OR 0.60 [0.29-1.22], respectively). A variant in *CD80* (rs16829984) was associated with increased prevalence of inhibitors compared to the reference allele (OR 1.73 [0.82-3.66]). With the exception of the mild hemophilia category for rs16829984, these associations were consistent across all severity categories and among enrollees with type 1 intron 22 inversions (Supplementary Table 3). The results for all other variants are reported in Supplementary Table 4.

The distribution of *HMOX1* $(GT)_n$ repeat (rs3074372) length among enrollees is shown in Supplementary Figure 1. While longer repeat length was associated with increased prevalence of inhibitors, this relationship was not statistically significant (OR 1.53 [0.85-2.77]) (Table 5). This relationship was consistent across severity categories and among enrollees with type 1 intron 22 inversions (Supplementary Table 5).

The distribution of IL10 $(CA)_n$ repeat (rs2234662) length among enrollees is shown in Supplementary Figure 2. Longer repeat length was associated with decreased prevalence of inhibitors (OR 0.66 [0.43-0.99]) (Table 6). With the exception of the mild hemophilia category,

this association was consistent across all severity categories and among enrollees with type 1 intron 22 inversions (Supplementary Table 6).

Discussion

Family studies have highlighted the importance of genetic risk factors other than the hemophilia-causing genotype for risk of inhibitor development among persons with hemophilia.[53] The results presented in this report confirm the importance of variation in the MHC region as a marker of inhibitor risk and identified several other genes outside the MHC region that may play a role in increasing or decreasing the risk of inhibitor development.

This investigation identified variants in the MHC region that may alter inhibitor risk. The rare MHC Class I *HLA-B**37 variant was associated with a markedly increased prevalence of inhibitors in this population. A recent meta-analysis summarized prior investigations of the associations between immune-response-related genetic variants and inhibitors among persons with hemophilia.[163] That report does not provide a summary of evidence for *HLA-B**37 because this variant has not been investigated in other studies of the association between MHC Class I variation and inhibitors. Only two studies have examined the relationship between *HLA-DPB1* genotype and inhibitors among PWH. One study[112], using restriction-fragment-length polymorphism mapping, found several variants in *HLA-DPB1* that were associated with increased risk of inhibitors, while the other study[82], using direct genotyping, found no association. The results of both the meta-analysis and our investigation provide evidence that genetic variation in the MHC region may play an important role in determining inhibitor risk.

This investigation also identified variants outside the MHC region that were associated with prevalence of inhibitors in this population. A variant in *IL1A* (rs17561) and a variant in *IL12B* (rs1003199) were associated with decreased prevalence of inhibitors, and a variant in *CD80* (rs16829984) was associated with increased prevalence of inhibitors compared to reference alleles. The adjusted confidence intervals for these variants cross the null. However,

permutation analyses indicated these variants were significantly associated with inhibitor prevalence. Similar to the MHC region variants described above, there is limited prior evidence for associations with these variants with inhibitor development among PWH. Astermark et al[102] included the *IL1A* variant on their large genotyping panel but found no consistent association in the populations they investigated. While Lozier et al[79] did not genotype the variant directly, their gene tagging approach identified a rare *IL1A* haplotype that was associated with increased risk for inhibitor development. Both Astermark et al[102] and Lozier et al[79] investigated the *IL12B* variant. Neither reported an association with the variant (or gene) and inhibitor development. Similarly, Astermark et al[102] investigated the variant in *CD80* and found no association. Because the number of variants investigated by Astermark et al[102] (over 13,000 were genotyped) fairly conservative criteria for identification of variants associated with inhibitors was used. Three different cohorts were genotyped and analyzed separately. Variants found to be significant predictors of inhibitors and yielding the same direction of effect in at least 2 of the 3 cohorts were considered for meta-analytic evaluation. It is possible the variants described above were significant predictors of inhibitors in at least one of the cohorts.

Much like the variants described above, limited evidence is available regarding the association with *HMOX1* $(GT)_n$ repeat (rs3074372) length and inhibitors among persons with hemophilia. Dimitrov et al[193] showed that induction of heme oxygenase reduced the onset of the anti-FVIII immune response in FVIII-deficient mice. Repesse et al[87] reported an increased risk of inhibitors associated with longer *HMOX1* $(GT)_n$ repeat lengths in a population of persons with severe hemophilia A. Longer repeat lengths have been shown to be associated with less production of heme oxygenase.[194] Our data also suggest an increased prevalence of inhibitors among enrollees with longer *HMOX1* $(GT)_n$ repeat lengths; however, this association was not statistically significant. This lack of significance could be due to limited statistical power to detect such an association. Bean et al[189] noted a striking difference in *HMOX1* $(GT)_n$ repeat length by race, with Blacks having longer repeat lengths than Whites. Our exclusion of Blacks

from this analysis due to sample size could have limited our ability to detect long repeat lengths that may be the most strongly associated with inhibitors.

The results of this investigation confirm other findings of a relationship between *IL10* microsatellite $(CA)_n$ repeat (rs2234662) length and inhibitors.[80,84,92,100] The meta-analysis[163] described above included four studies of *IL10* $(CA)_n$ repeat length and inhibitors, with an overall summary estimate that strongly suggested a reduced risk associated with longer repeat lengths (OR 0.58 [0.34-1.01]). Repeat *IL10* $(CA)_n$ repeat length may influence interleukin-10 production. Interleukin-10 is a pleiotropic cytokine that has been shown to suppress the systemic inflammatory response but also increase the secretion of immunoglobulins by activated B lymphocytes.[195-198]

This investigation attempted to identify genetic variants in the immune response pathway that may influence risk for inhibitors by mapping the MHC region and other genes in the immune response pathway as well as by evaluating the association between two repeat length polymorphisms that have been shown to alter gene expression (*HMOX1* rs3074372 and *IL10* rs2234662). A large, racially homogeneous sample of PWH was investigated, decreasing the effects of population stratification. However, even with a population in excess of 700 PWH, the power to detect statistically significant associations was limited due to the number of variants assessed, especially for rare variants that may be associated with the most drastic alterations in risk. In order to increase power, only independent variants not in linkage disequilibrium with nearby variants tested on the genotyping panel were evaluated. While this may have increased power to detect statistically significant associations, it reduced our ability to map genes completely, as differences in linkage disequilibrium could identify haplotype blocks within the population. Eliminating variants could decrease the ability to identify such haplotype blocks. Furthermore, while several variants known to alter gene expression and/or protein function were evaluated, the majority of variants were not expected to be direct markers of protein

production and were, instead, designed to identify particular genes that may be most related to inhibitor risk so that they can be evaluated more completely using other methodologies.

Conclusions

This investigation to identify variants in the immune response pathway that may be related to inhibitor development among PWH confirmed the importance of the MHC region in affecting risk for inhibitors and identified several novel variants in the immune response pathway that warrant further investigation. The study was limited by lack of power to detect associations related to rare genetic variants, highlighting the need for a coordinated approach to investigating the role of immune response genetics in risk of inhibitor development.

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Tables

	Inhibitor (-) N=596	Inhibitor (+) N=162
Age, mean (standard deviation)	22.56 (17.22)	24.97 (19.08)
Severity, n (%)		
Mild (FVIII:C > 5%)	137 (22.99)	13 (8.02)
Moderate (1% ≤ FVIII:C ≤ 5%)	96 (16.11)	14 (8.64)
Severe (FVIII:C < 1%)	363 (60.91)	119 (73.46)
Exposure Days at Enrollment, n (%)		
0-20	104 (17.45)	36 (22.22)
21-100	79 (13.26)	27 (16.67)
101-150	41 (6.88)	10 (6.17)
≥150	371 (62.25)	88 (54.32)
Hemophilia-Causing Variant, n (%)		
Missense	263 (44.13)	29 (17.9)
Nonsense	48 (8.05)	16 (9.88)
Frameshift	65 (10.91)	21 (12.96)
Splice Site	16 (2.68)	2 (1.23)
Inversion	163 (27.35)	70 (43.21)
Large Deletion	11 (1.85)	16 (9.88)
Large Duplication	3 (0.5)	1 (0.62)
Other	12 (2.01)	3 (1.85)
None	15 (2.52)	4 (2.47)

Table 1: Characteristics of Hemophilia Inhibitor Research Study enrollees included in investigation of associations between variants in immune-response-related genes and inhibitors among persons with hemophilia A.

FVIII:C: factor VIII activity

	All			Intron 22 Type 1 Inversion		
	Inhibitor (-) N=998*	**Inhibitor (+) N=258***	**OR (95% CI)**	**Inhibitor (-) N=230***	**Inhibitor (+) N=88***	**OR (95% CI)**
HLA-A						
*01	153 (15.3)	32 (12.4)	REF	35 (15.2)	14 (15.9)	REF
*02	297 (29.8)	73 (28.3)	1.18 (0.73-1.90)	75 (32.6)	22 (25)	0.73 (0.32-1.67)
*03	116 (11.6)	42 (16.3)	1.73 (1.00-2.99)	25 (10.9)	10 (11.4)	1.00 (0.36-2.74)
*11	63 (6.3)	21 (8.1)	1.59 (0.83-3.07)	20 (8.7)	7 (8)	0.88 (0.29-2.67)
*23	21 (2.1)	7 (2.7)	1.59 (0.60-4.26)	9 (3.9)	2 (2.3)	0.56 (0.10-3.16)
*24	89 (8.9)	22 (8.5)	1.18 (0.63-2.23)	23 (10)	8 (9.1)	0.87 (0.30-2.53)
*25	19 (1.9)	5 (1.9)	1.26 (0.41-3.82)	2 (0.9)	2 (2.3)	2.50 (0.29-21.69)
*26	27 (2.7)	2 (0.8)	0.35 (0.07-1.69)	8 (3.5)	1 (1.1)	0.31 (0.03-3.06)
*29	44 (4.4)	9 (3.5)	0.98 (0.42-2.30)	4 (1.7)	4 (4.5)	2.50 (0.51-12.33)
*30	34 (3.4)	12 (4.7)	1.69 (0.76-3.75)	4 (1.7)	6 (6.8)	3.75 (0.85-16.49)
*31	26 (2.6)	7 (2.7)	1.29 (0.49-3.38)	6 (2.6)	2 (2.3)	0.83 (0.14-5.06)
*32	39 (3.9)	9 (3.5)	1.10 (0.47-2.61)	4 (1.7)	3 (3.4)	1.87 (0.34-10.29)
*33	6 (0.6)	2 (0.8)	1.59 (0.28-8.98)	1 (0.4)	0 (0)	NE
*34	3 (0.3)	0 (0)	NE	2 (0.9)	0 (0)	NE
*36	1 (0.1)	0 (0)	NE	0 (0)	0 (0)	NE
*66	5 (0.5)	3 (1.2)	2.87 (0.60-13.61)	0 (0)	2 (2.3)	NE
*68	55 (5.5)	12 (4.7)	1.04 (0.48-2.25)	12 (5.2)	5 (5.7)	1.04 (0.29-3.73)
HLA-B						
*07	125 (12.53)	31 (12.02)	REF	26 (11.3)	13 (14.77)	REF
*08	106 (10.62)	20 (7.75)	0.76 (0.40-1.46)	28 (12.17)	10 (11.36)	0.71 (0.25-2.01)
*13	25 (2.51)	13 (5.04)	2.10 (0.93-4.75)	4 (1.74)	4 (4.55)	2.00 (0.40-10.07)
*14	32 (3.21)	13 (5.04)	1.64 (0.74-3.62)	10 (4.35)	4 (4.55)	0.80 (0.20-3.26)
*15	68 (6.81)	25 (9.69)	1.48 (0.79-2.80)	24 (10.43)	8 (9.09)	0.67 (0.22-1.99)
*18	49 (4.91)	13 (5.04)	1.07 (0.50-2.30)	8 (3.48)	3 (3.41)	0.75 (0.16-3.57)
*27	46 (4.61)	5 (1.94)	0.44 (0.15-1.26)	18 (7.83)	1 (1.14)	0.11 (0.01-1.03)
*35	81 (8.12)	23 (8.91)	1.14 (0.60-2.17)	23 (10)	5 (5.68)	0.43 (0.13-1.49)
*37	6 (0.6)	6 (2.33)	4.03 (1.14-14.20)	0 (0)	3 (3.41)	NE
*38	22 (2.2)	1 (0.39)	0.18 (0.02-1.57)	6 (2.61)	0 (0)	NE
*39	14 (1.4)	5 (1.94)	1.44 (0.46-4.55)	2 (0.87)	1 (1.14)	1.00 (0.07-13.71)
*40	83 (8.32)	23 (8.91)	1.12 (0.59-2.11)	11 (4.78)	6 (6.82)	1.09 (0.31-3.84)
*41	10 (1)	4 (1.55)	1.61 (0.45-5.84)	0 (0)	2 (2.27)	NE
*44	175 (17.54)	29 (11.24)	0.67 (0.37-1.20)	35 (15.22)	13 (14.77)	0.74 (0.28-1.96)
*45	6 (0.6)	4 (1.55)	2.69 (0.67-10.82)	0 (0)	0 (0)	NE
*47	2 (0.2)	0 (0)	NE	0 (0)	0 (0)	NE
*48	2 (0.2)	0 (0)	NE	0 (0)	0 (0)	NE
*49	14 (1.4)	5 (1.94)	1.44 (0.46-4.55)	3 (1.3)	2 (2.27)	1.33 (0.18-9.92)
*50	7 (0.7)	3 (1.16)	1.73 (0.39-7.59)	3 (1.3)	1 (1.14)	0.67 (0.06-7.96)
*51	55 (5.51)	19 (7.36)	1.39 (0.70-2.77)	12 (5.22)	7 (7.95)	1.17 (0.35-3.89)
*52	9 (0.9)	1 (0.39)	0.45 (0.05-4.09)	2 (0.87)	0 (0)	NE

*53	5 (0.5)	0 (0)	NE	1 (0.43)	0 (0)	NE
*55	12 (1.2)	1 (0.39)	0.34 (0.04-2.98)	4 (1.74)	0 (0)	NE
*56	4 (0.4)	1 (0.39)	1.01 (0.10-10.46)	2 (0.87)	0 (0)	NE
*57	34 (3.41)	13 (5.04)	1.54 (0.70-3.39)	6 (2.61)	5 (5.68)	1.67 (0.40-6.97)
*58	6 (0.6)	0 (0)	NE	2 (0.87)	0 (0)	NE
HLA-C						
*01	26 (2.61)	4 (1.56)	REF	6 (2.61)	0 (0)	REF
*02	50 (5.02)	13 (5.04)	1.69 (0.47-6.07)	19 (8.27)	5 (5.69)	NE
*03	131 (13.13)	40 (15.51)	1.98 (0.62-6.38)	35 (15.22)	12 (13.64)	NE
*04	105 (10.53)	28 (10.86)	1.73 (0.53-5.70)	28 (12.18)	9 (10.23)	NE
*05	103 (10.33)	22 (8.53)	1.39 (0.42-4.64)	18 (7.83)	8 (9.1)	NE
*06	82 (8.22)	37 (14.35)	2.93 (0.90-9.54)	15 (6.53)	11 (12.5)	NE
*07	307 (30.77)	67 (25.97)	1.42 (0.45-4.44)	72 (31.31)	28 (31.82)	NE
*08	33 (3.31)	12 (4.66)	2.36 (0.64-8.73)	9 (3.92)	3 (3.41)	NE
*12	57 (5.72)	12 (4.66)	1.37 (0.38-4.95)	14 (6.09)	2 (2.28)	NE
*14	16 (1.61)	2 (0.78)	0.81 (0.12-5.43)	3 (1.31)	0 (0)	NE
*15	27 (2.71)	6 (2.33)	1.44 (0.34-6.13)	5 (2.18)	3 (3.41)	NE
*16	51 (5.12)	11 (4.27)	1.40 (0.38-5.15)	6 (2.61)	5 (5.69)	NE
*17	10 (1.01)	4 (1.56)	2.60 (0.50-13.48)	0 (0)	2 (2.28)	NE

Table 2: Associations between inhibitor prevalence and MHC Class I alleles among enrollees in the Hemophilia Inhibitor Research Study.

*Allele frequency

OR: odds ratio

95% CI: 95% Confidence Interval

REF: reference group

NE: not estimatable

	All			Intron 22 Type 1 Inversion		
	Inhibitor (-) N=998*	Inhibitor (+) N=258*	OR (95% CI)	Inhibitor (-) N=230*	Inhibitor (+) N=88*	OR (95% CI)
HLA-DPB1						
*01	57 (5.71)	6 (2.33)	REF	18 (7.83)	2 (2.27)	REF
*02	143 (14.33)	38 (14.73)	2.52 (0.97-6.60)	23 (10)	13 (14.77)	5.09 (0.94-27.67)
*03	116 (11.62)	32 (12.4)	2.62 (0.99-6.95)	23 (10)	8 (9.09)	3.13 (0.54-18.07)
*04	532 (53.31)	141 (54.65)	2.52 (1.02-6.23)	134 (58.26)	47 (53.41)	3.16 (0.65-15.24)
*05	31 (3.11)	9 (3.49)	2.76 (0.85-8.97)	8 (3.48)	1 (1.14)	1.13 (0.08-16.25)
*09	13 (1.3)	4 (1.55)	2.92 (0.67-12.75)	3 (1.3)	1 (1.14)	3.00 (0.18-50.90)
*10	23 (2.3)	6 (2.33)	2.48 (0.68-9.04)	3 (1.3)	4 (4.55)	12.00 (1.33-108.13)
*11	29 (2.91)	4 (1.55)	1.31 (0.32-5.37)	3 (1.3)	2 (2.27)	6.00 (0.53-68.00)
*13	16 (1.6)	7 (2.71)	4.16 (1.15-15.04)	4 (1.74)	5 (5.68)	11.25 (1.43-88.78)
*14	10 (1)	2 (0.78)	1.9 (0.31-11.78)	5 (2.17)	1 (1.14)	1.80 (0.12-27.59)
*15	6 (0.6)	1 (0.39)	1.58 (0.14-17.35)	0 (0)	0 (0)	NE
*16	8 (0.8)	0 (0)	NE	3 (1.3)	0 (0)	NE
*17	9 (0.9)	5 (1.94)	5.28 (1.24-22.5)	1 (0.43)	2 (2.27)	18.00 (0.94-345.10)
*19	5 (0.5)	3 (1.16)	5.70 (1.00-32.64)	2 (0.87)	2 (2.27)	9.00 (0.69-117.5)
HLA-DQA1						
*01	412 (41.28)	111 (43.02)	REF	100 (43.48)	36 (40.91)	REF
*02	137 (13.73)	43 (16.67)	1.16 (0.76-1.78)	23 (10)	17 (19.32)	2.05 (0.95-4.44)
*03	173 (17.33)	36 (13.95)	0.77 (0.50-1.20)	48 (20.87)	15 (17.05)	0.87 (0.42-1.8)
*04	21 (2.1)	5 (1.94)	0.88 (0.31-2.52)	7 (3.04)	3 (3.41)	1.19 (0.27-5.21)
*05	251 (25.15)	62 (24.03)	0.92 (0.64-1.32)	51 (22.17)	17 (19.32)	0.93 (0.46-1.87)
*06	4 (0.4)	1 (0.39)	0.93 (0.09-9.38)	1 (0.43)	0 (0)	NE
HLA-DQB1						
*02	228 (22.85)	52 (20.16)	0.88 (0.54-1.42)	45 (19.57)	17 (19.32)	1.16 (0.49-2.75)
*03	343 (34.37)	90 (34.88)	1.01 (0.65-1.57)	80 (34.78)	32 (36.36)	1.23 (0.57-2.64)
*04	21 (2.1)	4 (1.55)	0.73 (0.23-2.39)	7 (3.04)	2 (2.27)	0.88 (0.15-5.15)
*05	154 (15.43)	40 (15.5)	REF	43 (18.7)	14 (15.91)	REF
*06	252 (25.25)	72 (27.91)	1.10 (0.70-1.74)	55 (23.91)	23 (26.14)	1.28 (0.57-2.90)
HLA-DRB1						
*01	108 (10.82)	25 (9.69)	REF	32 (13.91)	8 (9.09)	REF
*03	132 (13.23)	25 (9.69)	0.82 (0.43-1.55)	30 (13.04)	6 (6.82)	0.80 (0.23-2.74)
*04	159 (15.93)	35 (13.57)	0.95 (0.52-1.73)	44 (19.13)	15 (17.05)	1.36 (0.49-3.79)
*07	136 (13.63)	42 (16.28)	1.33 (0.74-2.39)	25 (10.87)	16 (18.18)	2.56 (0.90-7.30)
*08	27 (2.71)	5 (1.94)	0.80 (0.27-2.41)	9 (3.91)	3 (3.41)	1.33 (0.27-6.58)
*09	16 (1.6)	1 (0.39)	0.27 (0.03-2.37)	3 (1.3)	0 (0)	NE
*10	6 (0.6)	3 (1.16)	2.16 (0.47-9.94)	1 (0.43)	3 (3.41)	12.00 (0.97-148.28)
*11	91 (9.12)	27 (10.47)	1.28 (0.67-2.44)	15 (6.52)	6 (6.82)	1.60 (0.44-5.79)
*12	15 (1.5)	4 (1.55)	1.15 (0.33-4.01)	4 (1.74)	3 (3.41)	3.00 (0.51-17.64)
*13	118 (11.82)	41 (15.89)	1.50 (0.83-2.71)	27 (11.74)	11 (12.5)	1.63 (0.54-4.89)
*14	30 (3.01)	11 (4.26)	1.58 (0.67-3.74)	5 (2.17)	2 (2.27)	1.60 (0.24-10.76)
*15	146 (14.63)	37 (14.34)	1.09 (0.60-1.98)	31 (13.48)	14 (15.91)	1.81 (0.63-5.16)
*16	14 (1.4)	2 (0.78)	0.62 (0.12-3.13)	4 (1.74)	1 (1.14)	1.00 (0.09-11.51)

Table 3: Associations between inhibitor prevalence and MHC Class II alleles among enrollees in the Hemophilia Inhibitor Research Study.

*Allele frequency

OR: odds ratio

95% CI: 95% Confidence Interval

REF: reference group

NE: not estimatable

	All			Intron 22 Type 1 Inversion		
	Inhibitor (-) N=508	**Inhibitor (+) N=134**	**OR (95% CI)**	**Inhibitor (-) N=115**	**Inhibitor (+) N=46**	**OR (95% CI)**
***IL1A* rs17561**						
CC	233 (45.96)	79 (59.4)	REF	54 (46.96)	29 (64.44)	REF
AC	228 (44.97)	46 (34.59)	0.58 (0.30-1.14)	52 (45.22)	14 (31.11)	0.49 (0.14-1.68)
AA	46 (9.07)	8 (6.02)		9 (7.83)	2 (4.44)	
***IL12B* rs1003199**						
GG	121 (23.82)	46 (34.33)	REF	29 (25.22)	17 (36.96)	REF
GA	256 (50.39)	58 (43.28)	0.60 (0.29-1.22)	54 (46.96)	19 (41.3)	0.58 (0.16-2.04)
AA	131 (25.79)	30 (22.39)		32 (27.83)	10 (21.74)	
***CD80* rs16829984**						
GG	397 (79.4)	89 (68.99)	REF	32 (28.07)	32 (72.73)	REF
CG	95 (19)	37 (28.68)	1.73 (0.82-3.66)	10 (8.77)	10 (22.73)	2.30 (0.53-9.99)
CC	8 (1.6)	3 (2.33)		2 (1.75)	2 (4.55)	

Table 4: Top candidate associations between inhibitor prevalence and variants in genes in the immune response pathway among enrollees in the Hemophilia Inhibitor Research Study.

OR: odds ratio

95% CI: 95% Confidence Interval

REF: reference group

	All			Intron 22 Type 1 Inversion		
	Inhibitor (-) N=595	Inhibitor (+) N=161	OR (95% CI)	Inhibitor (-) N=130	Inhibitor (+) N=58	OR (95% CI)
***HMOX1* Genotype**						
SS	86(14.45)	16(9.94)	REF	15(11.54)	5(8.62)	REF
SL	261(43.87)	67(41.61)	1.53 (0.85-2.77)	61(46.92)	25(43.1)	1.38 (0.45-4.23)
LL	248(41.68)	78(48.45)		54(41.54)	28(48.28)	

Table 5: Association between inhibitor prevalence and *HMOX1* $(GT)_n$ repeat (rs3074372) length among enrollees in the Hemophilia Inhibitor Research Study.

OR: odds ratio

95% CI: 95% Confidence Interval

REF: reference group

	All			Intron 22 Type 1 Inversion		
	Inhibitor (-) N=563	Inhibitor (+) N=151	OR (95% CI)	Inhibitor (-) N=124	Inhibitor (+) N=55	OR (95% CI)
***IL10* Genotype**						
SS	132 (23.45)	48 (31.79)	REF	25 (20.16)	19 (34.55)	REF
SL	309 (54.88)	62 (41.06)	0.66 (0.43-0.99)	75 (60.48)	22 (40)	0.48 (0.23-1.01)
LL	122 (21.67)	41 (27.15)		24 (19.35)	14 (25.45)	

Table 6: Association between inhibitor prevalence and *IL10* microsatellite $(CA)_n$ repeat (rs2234662) length among enrollees in the Hemophilia Inhibitor Research Study.

OR: odds ratio

95% CI: 95% Confidence Interval

REF: reference group

Chapter 6: Evaluation of inhibitor risk prediction tools based on genetic risk factors in persons with hemophilia A

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Abstract

Inhibitors are the most important treatment-related complication among persons with hemophilia. Currently, the only validated risk prediction tool relies on information about prior product exposure, limiting its utility to predict inhibitor risk in untreated or minimally-treated patients. A risk prediction tool based on patient-related risk factors that could be available before initiation of treatment could be more useful clinically. This investigation evaluates six risk prediction tools based on genetic risk factors for inhibitors among persons with hemophilia A. The tools combine information on genotypes for variants in genes in the immune response pathway and the hemophilia genotype. Variants in genes in the immune response pathway were weighted based on 1) estimates from a meta-analysis or 2) results of a prior investigation in this population and combined with 3 different hemophilia genotype categorization schemes, for a total of 6 tools evaluated. The ability of each tool to correctly predict inhibitor status was evaluated in 558 White participants enrolled in the Hemophilia Inhibitor Research Study with complete genetic information. Tool performance was evaluated by examining the area under the receiver operating curve (AUC) after cross-validation a using logistic regression model. The tool

that combined information on immune-response genes previously-found to be associated with inhibitors in this population and categorized the hemophilia genotype based on previously-reported estimates of effect performed best (AUC=0.75 and AUC=0.62 among persons with mild/moderate and severe disease, respectively). The variants in genes in the immune response pathway included in this tool have not been validated in other populations. Although cross-validation methods were used to reduce bias introduced by using the same data to simultaneously fit and evaluate the models, validation of these findings in other populations is warranted.

Introduction

Hemophilia A (HA), a bleeding disorder caused by loss or dysfunction of the pro-coagulant protein factor VIII (FVIII), currently affects approximately 20,000 males in the United States.[1] The missing or dysfunctional FVIII is often replaced using plasma-derived or recombinant FVIII protein.[185] Antibody development against these replacement proteins has emerged as the most important treatment-related complication among persons with HA. These antibodies (inhibitors) develop in 10%-15%[2] of all persons with HA and are associated with increased risk of early mortality[4], product utilization[3], and healthcare costs[3,5,39].

The only validated inhibitor risk prediction tool was developed and evaluated among persons with severe hemophilia A.[7] The tool assigns a risk score based on family history of inhibitor development, type of HA-causing genetic defect, and intensity of first treatment product exposure. Criticisms of this tool have included the reliance on prior treatment product exposure[8], limiting its utility for use in untreated or minimally-treated persons with HA. A tool based only on patient genetic characteristics could be more useful clinically. Understanding which patients may be most likely to develop inhibitors may help guide treatment decisions, such as avoiding intensive factor replacement therapy that may induce “danger signals” that upregulate immune response or delaying use of recombinant FVIII products during the first few exposure days.[62-65]

We have recently evaluated a series of tools that categorize the hemophilia genotype in order to predict inhibitor risk.[199] None of them performed better than the previously-validated tool discussed above.[8] Variants in genes in the immune response pathway have also been reported to contribute to patient-related risk factors for inhibitor development.[8] Our recent meta-analysis[163] and investigation[200] of these genes highlight the importance of variation in the immune response pathway in affecting inhibitor risk. The goal of this study is to evaluate a series of tools that incorporate genetic information from both the immune response pathway and the hemophilia genotype in order to predict inhibitor risk among persons with HA.

Methods

Population

A subset of participants enrolled in the Hemophilia Inhibitor Research Study (HIRS) were included in this investigation. Extensive details of HIRS are provided elsewhere.[9,130] Briefly, HIRS was a pilot study for national inhibitor surveillance conducted at 17 participating hemophilia treatment centers. Participants were tested at baseline, annually, and upon clinical suspicion for inhibitors and submitted blood specimens for genotyping. Study coordinators collected demographic, treatment, and inhibitor history data using a standard data collection tool. For the current study, data from participants with HA who did not refuse genetic testing and who had complete genotyping information were analyzed. Data from the first-enrolled were used when relatives were co-enrolled in HIRS. Only White, Non-Hispanic participants were included in order to control for the potential impact of race.

Genotyping

Hemophilia genotyping was completed as previously described.[9] The *F8* gene was sequenced in forward and reverse directions using the VariantSEQr™ protocol and analyzed on a 3730 DNA Analyzer (Applied Biosystems, Carlsbad, CA, USA). Inversions of the *F8* gene were detected using PCR.[67,68] Large duplications were detected using multiplex ligation-dependent probe amplification (SALSA MLPA Kit P178-A1, MRC Holland, Amsterdam, The Netherlands).[132]

Variants in the immune response pathway were genotyped using a variety of methods, as previously described.[200] Results of a fine mapping of the major histocompatibility (MHC) region using a GoldenGate genotyping panel (Illumina, San Diego, CA, US) were used to impute human leukocyte antigen (HLA) genotype.[190] Single nucleotide variants in genes in the immune response pathway outside the MHC region were genotyped using the same genotyping panel. Variants were chosen based on their ability to map genes of interest by tagging haplotype blocks or because they were previously-reported to be associated with inhibitors. *HMOX1* promoter $(GT)_n$ dinucleotide repeat (rs3074372) length and *IL10* microsatellite $(CA)_n$ repeat (rs2234662) length were genotyped using fragment analysis, as previously described.[188,189,200] *HMOX1* $(GT)_n$ repeat sizes were grouped as small (S) (≤ 25 repeats) and large (L) (> 25 repeats). *IL10* $(CA)_n$ repeat sizes were grouped as small (S) (≤ 18 repeats) and large (L) (> 18 repeats).

Risk Prediction Tools

A series of risk prediction tools that combined information on both the hemophilia genotype and information on variants in genes in the immune response pathway were evaluated. A description of each component is provided below.

Hemophilia Genotype Scoring

This group previously evaluated the ability of 3 different inhibitor risk prediction tools based on hemophilia genotype data to accurately assign inhibitor status in HIRS participants.[199] The details of each tool are discussed in more detail in that report. The first tool (Pathogenicity Tool) assigned each hemophilia genotype a score based on predicted pathogenicity, as defined using the American College of Medical Genetics guidelines.[133] The ACMG guidelines outline criteria to use to categorize variants as pathogenic, likely pathogenic, unknown significance, likely benign, and benign. The criteria are graded ‘very strong’, ‘strong’, ‘moderate’, and ‘supporting’. Each criteria was assigned a point value. Points were summed across all evidence categories to produce a final variant score. The second tool (Function Tool) assigned each hemophilia genotype a score based on predicted impact on gene function, with 4 points being

assigned for variants predicted to produce no gene product (multi-exon deletions), 3 points for variants predicted to produce a possible gene product (inversions, single-exon deletions, large duplications, nonsense variants, splice-altering variants, and frameshift variants), 2 points for predicted immunogenic missense variants (missense variants at known epitope locations), and 1 point for missense variants outside of known inhibitor epitope locations. The third tool (Evidence-based Tool) was a severity-specific tool that assigned each hemophilia genotype a score based on previously-published[10,31] estimates of effect of variant types on inhibitor risk among persons with mild/moderate and severe HA, respectively.

Immune Response Variant Scoring

Two different mechanisms for summarizing risk related to immune-response-gene variation were evaluated. In both scores were computed by multiplying the estimate of effect for a particular variant by 1 if the participant carries at least one alternate allele and 0 if not and summing across all variants included in the score. The first assigned an immune response variant score based on estimates of effect derived from the results of a recent meta-analysis.[163] In that meta-analysis 5 variants were identified as being statistically significantly associated with inhibitors, including *HLA-DQB1**06, *HLA-DRB1**15, rs1800629 in *TNFA*, rs1800896 in *IL10*, and *IL10* $(CA)_n$ repeat length. Details of score assignment are outlined in Supplementary Table 1. The second assigned an immune response variant score based on estimates of effect derived from an analysis of immune-response-related variants conducted in the HIRS population.[200] In that analysis 8 variants were associated with inhibitors in the HIRS population, including *IL10* $(CA)_n$ repeat length, rs17561 in *IL1A*, rs1003199 in *IL12B*, rs16829984 in *CD80*, *HLA-DPB1**04, *HLA-DPB1**13, *HLA-DPB1**17, and *HLA-DPB1**19. Details of score assignment are outlined in Supplementary Table 2.

A total of 6 tools were evaluated, each a combination of one of the 3 hemophilia genotype scores and one of the two immune response variant scores: 1. pathogenicity score and meta-analysis-based estimates; 2. function score and meta-analysis-based estimates; 3. evidence-based score

and meta-analysis-based estimates; 4. pathogenicity score and HIRS-based estimates; 5. function score and HIRS-based estimates; and 6. evidence-based score and HIRS-based estimates.

Statistics

Differences between scores among inhibitor-positive and inhibitor-negative participants were evaluated using the Wilcoxon rank sum test. The ability of each of the tools to correctly predict inhibitor status was evaluated using the area under the receiver operating curve (AUC) using a logistic regression model. Because the HIRS-based estimates were derived using data from the same cohort, cross-validation was used to minimize bias introduced by using the same data to simultaneously fit and evaluate models including these estimates using the 'crossvalidate' option within the PROC LOGISTIC procedure. All analyses were done using SAS, version 9.4.

Results

Among 1,300 HIRS participants, 758 were unrelated, non-Hispanic White persons with HA. Of these, 558 had complete genotyping information. A description of participants included in the analysis and a comparison to the overall White, HA HIRS population is provided in Table 1. The analytic population was not appreciably different than the overall population of White enrollees with HA. The majority of eligible participants had severe disease and had over 150 historical exposure days at study enrollment. Missense variants were the most common cause of HA, followed by inversions.

The ability of tools that categorize the hemophilia-causing variant to accurately predict inhibitor status has been previously investigated by this group.[199] The evidence-based tool that scored variants based on previously-published estimates of variant category effect performed best (AUC = 0.67 and AUC = 0.61 for persons with mild/moderate and severe disease, respectively) (Table 2).

The distributions of immune response variant scores using the meta-analytic and HIRS-based estimates are shown in Figure 1. The score using meta-analytic based estimates was lower among inhibitor-positive participants (Figure 1A), though only statistically significantly among participants with severe disease. The immune response variant score based on HIRS estimates of effect was statistically significantly higher among inhibitor-positive participants (Figure 1B). Though neither of these scores was able to consistently predict inhibitor status, the score using HIRS-based estimates of effect performed better than the score using meta-analytic based estimates of effect (Table 2). The cross-validation results indicate that the results may overstate the performance of the tool using HIRS-based estimates, as the AUC after cross-validation was significantly lower (Figure 2).

The performances of risk prediction tools that combine hemophilia genotype score information and immune response variant score information are outlined in Table 2 and Figures 3 and 4. Among tools that used the immune response variant score based on meta-analytic derived estimates of effect, the tool that categorized the hemophilia genotype based on previously-published evidence (Figure 3C) performed best (AUC=0.72 and AUC=0.63 for persons with mild/moderate and severe disease, respectively). This increased the ability to predict inhibitor status compared to using information on the hemophilia genotype alone (Table 2). Among tools that used the immune response variant score based on HIRS estimates of effect, again, the tool that categorized the hemophilia genotype based on previously-published evidence (Figure 4C) performed best (AUC=0.80 and AUC=0.65 for persons with mid/moderate and severe disease, respectively) (Table 2). The cross-validation results were attenuated (AUC=0.75 and AUC=0.62 for persons with mild/moderate and severe disease, respectively). This tool performed better than the tool that used the immune response variant score based on meta-analytic estimates of effect.

Discussion

The only validated tool to predict inhibitor risk among PWH[7] has several drawbacks, including only being validated in persons with severe disease and its reliance on prior treatment.[8] A risk prediction tool based on patient characteristics that could be used in patients with all types of disease severity prior to initiation of treatment could be more useful clinically. The tools evaluated in this investigation used information on the hemophilia-causing genotype and variation in genes in the immune response pathway. While none performed better than the previously-validated tool where the AUC was 0.74[7] among persons with severe disease, one tool performed well among persons with mild/moderate disease (AUC=0.75).

A previous analysis by this group investigating the feasibility of predicting inhibitor risk based on the hemophilia genotype alone indicated doing so was not useful.[199] The results presented here indicate that the addition of information on variation in genes in the immune response pathway enhances the ability to predict inhibitor risk. Inhibitor development is a T-cell dependent response where foreign FVIII is taken up by antigen-presenting cells and presented to T-helper cell receptors by MHC class II proteins.[6] The MHC class II-T cell receptor interaction, along with costimulatory signals from cytokine proteins, promotes B-cell maturation into antibody-secreting plasma cells.[6] It follows that variation in genes integral to this process, such as MHC class II proteins or cytokines, that influence protein function or that are markers for variants that influence protein function may influence inhibitor risk. This is supported by the observation that inclusion of information on variants in genes in the immune response pathway that were previously shown to be associated with inhibitors in PWH into a genetic risk prediction tool enhanced the performance of the tool.

Several of the tools evaluated performed best among persons with mild/moderate disease. This is likely because tools to predict inhibitor risk based only on the hemophilia genotype generally performed better among participants with mild/moderate disease.[199] Furthermore, these tools were generally better able to assign inhibitor risk than immune response variation scores alone.

Two different mechanisms to classify variation in genes in the immune response pathway were evaluated. The first, using estimates of effect derived from a previously-conducted meta-analysis[163] of variants in immune response genes and their association with inhibitors in PWH, did not perform as well as the second, using estimates of effect derived from an analysis of variants in immune response genes and their association with inhibitors conducted using the HIRS population[200]. This is not surprising, as the estimates of effect used to produce the score were derived in the same population. Cross-validation methods were used to attempt to reduce the bias introduced by using the same data to fit and evaluate the tool. Perhaps more importantly, the variants identified in the HIRS analysis have not been validated in any other population. Future work to validate the association of these variants with inhibitors and their ability to predict inhibitor risk in a different population is needed. A validation study by Hashemi et al found that the previously-validated inhibitor risk prediction tool showed a worse discrimination when evaluated in a different population, highlighting the importance of validation in an external population.[126]

Only 74% of participants enrolled in HIRS had complete genotype information and were eligible for this analysis. The comparison to the population of White participants with HA enrolled in HIRS indicates that the analytic sample was not appreciably different than the overall population, indicating this sub-selection did not likely introduce bias. Furthermore, comparison of the HIRS population to the population of participants in the Universal Data Collection program[201], a bleeding disorder surveillance system enrolling participants from 140 hemophilia treatment centers across the United States, during the same time period indicates HIRS participants are similar to the general hemophilia A population in the distribution of severity and age. Since the analytic sample was restricted to White, non-Hispanic participants with hemophilia A, it remains a question as to how well the tools presented in this report would perform in non-White populations or among persons with hemophilia B. Because linkage disequilibrium structure is likely to differ between race and ethnic populations, variants that are

associated with inhibitors among Whites may not necessarily be associated with inhibitors in other populations – especially if the variants are tagging important gene regions and are not themselves causative of increased or decreased protein function.

Conclusions

The ability to predict inhibitor status based on genetic characteristics was increased by including information on both the hemophilia genotype and variation in genes in the immune response pathway. The best-performing tool did not perform as well as a tool that requires information about prior treatment among persons with severe disease but performed well among persons with mild/moderate disease.

Tables

	Analytic Sample		HIRS Hemophilia A Population	
	Inhibitor (-) N=450 (81%)	**Inhibitor (+) N=108 (19%)**	**Inhibitor (-) N=596 (79%)**	**Inhibitor (+) N=162 (21%)**
Age, mean (standard deviation)	22.67 (17.0)	25.44 (18.9)	22.56 (17.2)	24.97 (19.1)
Severity, n (%)				
Mild (FVIII:C > 5%)	100 (22.2)	10 (9.3)	137 (23.0)	13 (8.0)
Moderate (1% ≤ FVIII:C ≤ 5%)	68 (15.1)	11 (10.2)	96 (16.1)	14 (8.6)
Severe (FVIII:C < 1%)	252 (56.0)	80 (74.1)	363 (60.9)	119 (73.5)
Exposure Days at Enrollment, n (%)				
0-20	78 (17.3)	21 (19.4)	104 (17.5)	36 (22.2)
21-100	45 (10.0)	13 (12.0)	79 (13.3)	27 (16.7)
101-150	36 (8.0)	9 (8.3)	41 (6.9)	10 (6.2)
≥150	290 (64.4)	65 (60.2)	371 (62.3)	88 (54.3)
Hemophilia-Causing Variant, n (%)				
Missense	195 (43.3)	24 (22.2)	263 (44.1)	29 (17.9)
Nonsense	38 (8.4)	10 (9.3)	48 (8.1)	16 (9.9)
Frameshift	48 (10.7)	13 (12.0)	65 (10.9)	21 (13.0)
Splice Site	14 (3.1)	2 (1.9)	16 (2.7)	2 (1.2)
Inversion	129 (28.7)	44 (40.7)	162 (27.2)	70 (43.2)
Large Deletion	7 (1.6)	13 (12.0)	11 (1.9)	16 (9.9)
Large Duplication	3 (0.7)	0 (0.0)	3 (0.5)	1 (0.6)
Other	8 (1.8)	2 (1.9)	13 (2.2)	3 (1.9)
None	8 (1.8)	0 (0.0)	15 (2.5)	4 (2.5)

Table 1: Characteristics of Hemophilia Inhibitor Research Study participants included in an evaluation of tools to predict inhibitor risk based on genetic risk factors for inhibitor development and comparison to all participants with hemophilia A enrolled in the study.

FVIII:C: factor VIII activity

	Receiver Operating Curve Area		
	All	**Mild/Moderate**	**Severe**
Hemophilia Variant Categorization			
Pathogenicity Tool	0.55	0.57	0.50
Function Tool	0.65	0.52	0.58
Evidence-Based Tool		0.67	0.61
Immune Response Variant Estimates			
Meta-Analysis Estimates	0.56	0.55	0.58
Prior Analysis Estimates	0.63	0.67	0.61
Hemophilia Variant Categorization + Immune Response Variant Estimates			
Pathogenicity Tool + Meta-Analysis Estimates	0.58	0.67	0.59
Function Tool + Meta-Analysis Estimates	0.65	0.53	0.63
Evidence-Based Tool + Meta-Analysis Estimates		0.72	0.63
Pathogenicity Tool + HIRS-Based Estimates	0.64	0.76	0.61
Function Tool + HIRS-Based Estimates	0.69	0.68	0.65
Evidence-Based Tool + HIRS-Based Estimates		0.80	0.65

Table 2: Receiver Operating Curve Area estimates for models based on information about the hemophilia genotype, variation in genes in the immune response pathway, and a combination of the two among the participants Hemophilia Inhibitor Research Study, overall and by severity.

Figures

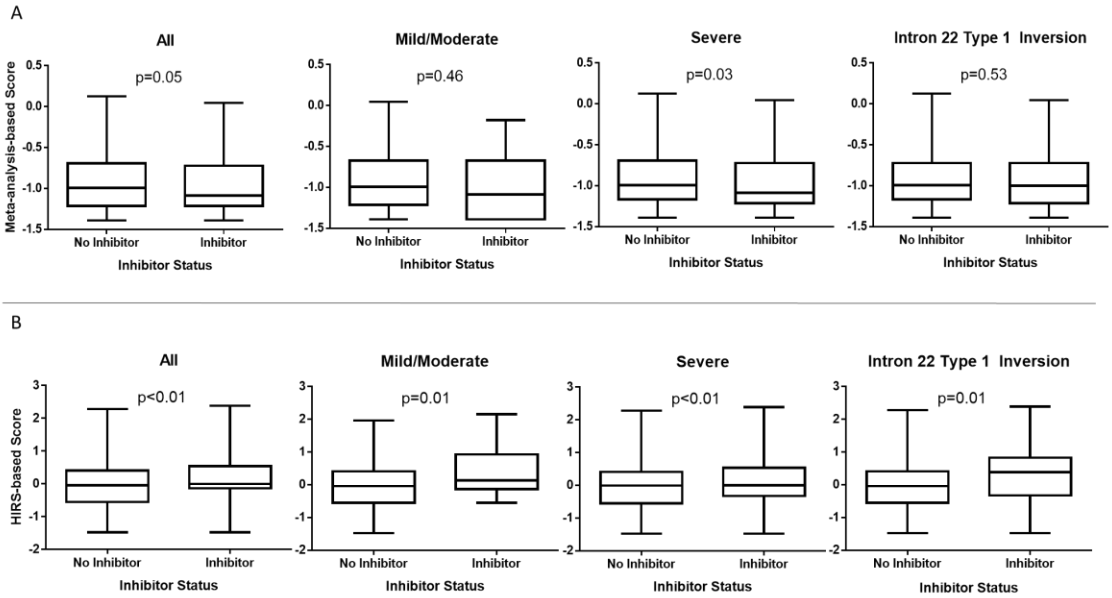

Figure 1: Distribution of immune response variant scores assigned based on (A) meta-analytic estimates of effect and (B) estimates of effect derived from an analysis of associations between variants in genes in the immune response pathway and inhibitors among participants in the Hemophilia Inhibitor Research Study, overall and by hemophilia severity.

p: Wilcoxon rank sum test

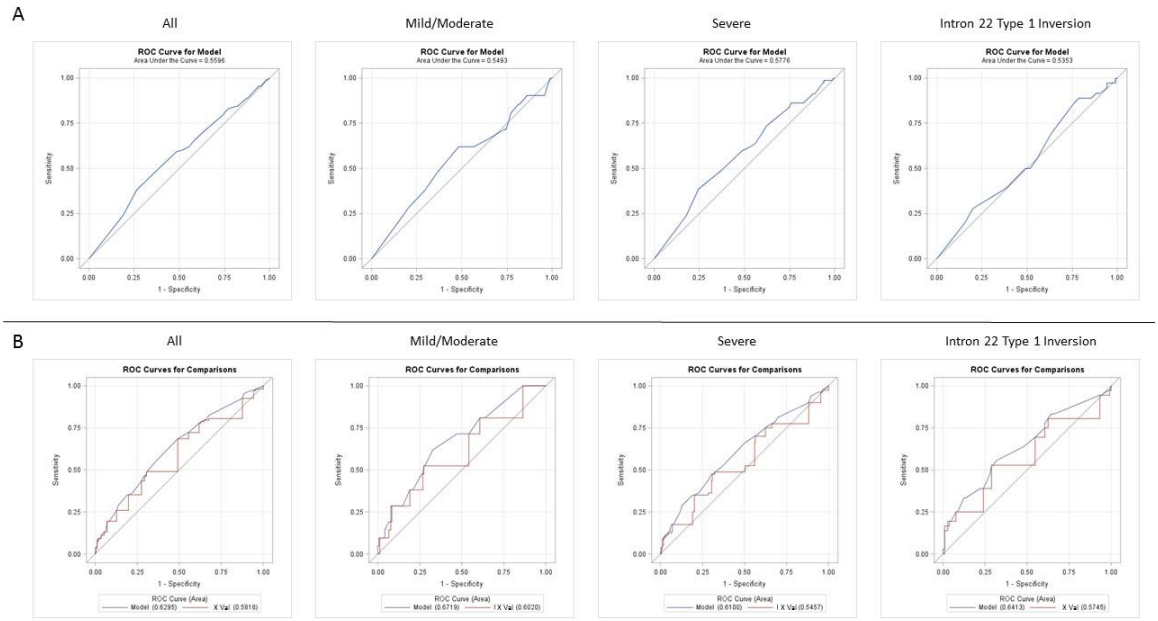

Figure 2: Evaluating ability of immune response variant scores assigned based on (A) meta-analytic estimates of effect and (B) estimates of effect derived from an analysis of associations between variants in genes in the immune response pathway and inhibitors to correctly assign inhibitor risk among participants in the Hemophilia Inhibitor Research Study, overall and by hemophilia severity.

ROC: receiver operating characteristic

X Val: cross-validation

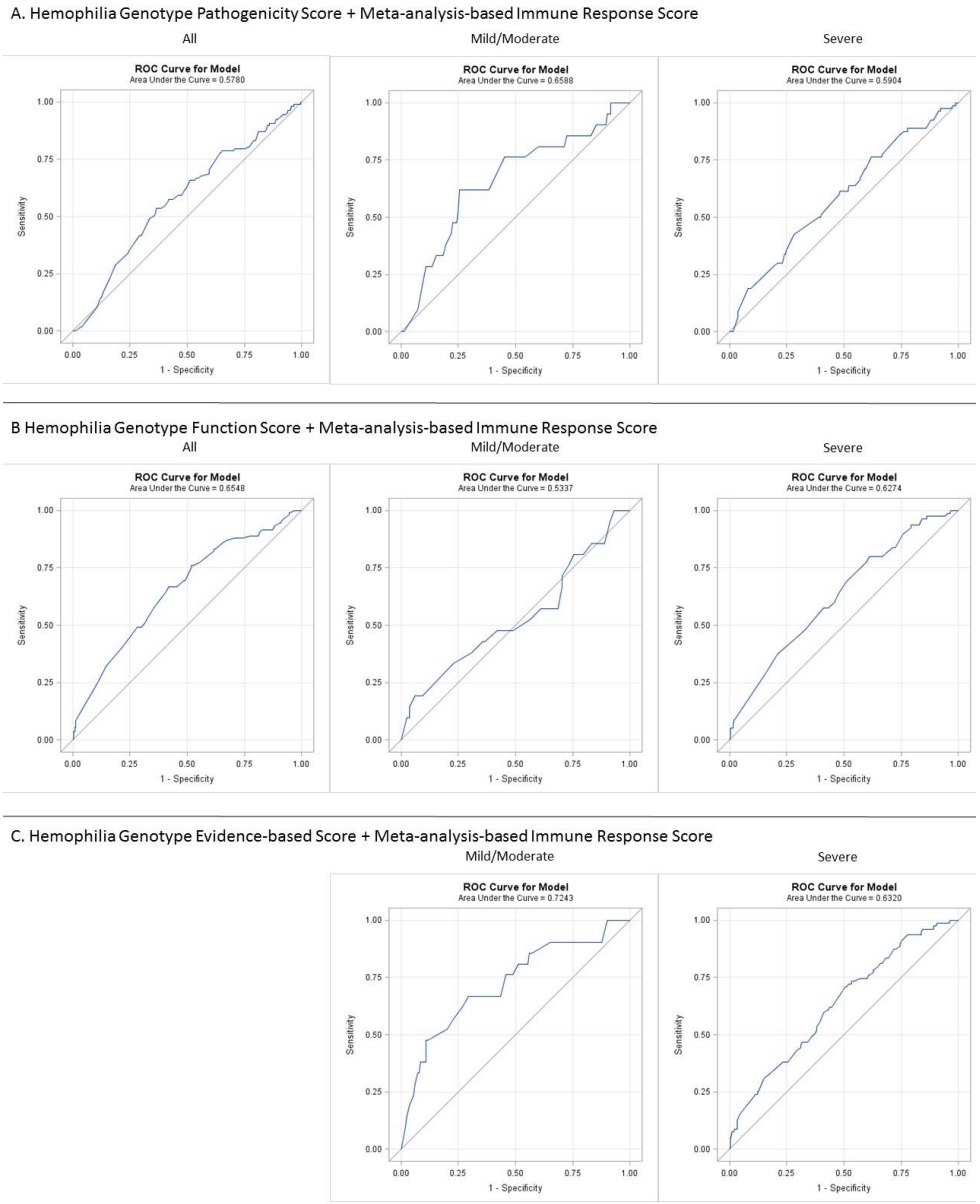

Figure 3: Comparison performance of inhibitor risk prediction tools that combine information on hemophilia genotype and variation in genes in the immune response pathway among participants enrolled in the Hemophilia Inhibitor Research Study (HIRS), overall and by hemophilia severity. A) Tool that used the immune response variant score derived from meta-analytic estimates and hemophilia genotype score based on predicted pathogenicity. B) Tool that used

the immune response variant score derived from meta-analytic estimates and hemophilia genotype score based on predicted impact on gene function. C) Tool that used the immune response variant score derived from meta-analytic estimates and hemophilia genotype score based on previously-published evidence of effect of variant category.

ROC: receiver operating characteristic

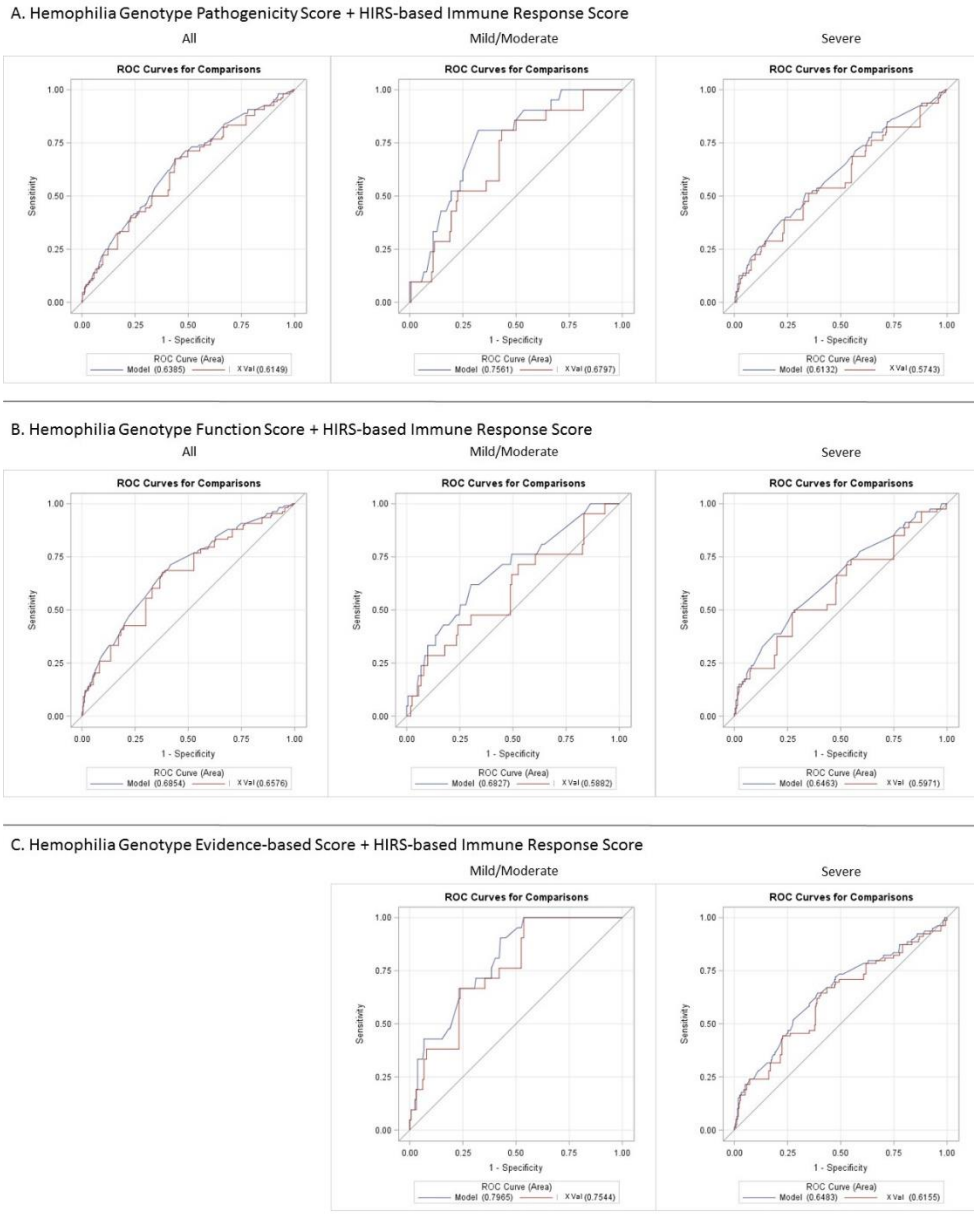

Figure 4: Comparison performance of inhibitor risk prediction tools that combine information on hemophilia genotype and variation in genes in the immune response pathway among participants enrolled in the Hemophilia Inhibitor Research Study (HIRS), overall and by hemophilia severity. A) Tool that used the immune response variant score derived from HIRS analysis estimates and hemophilia genotype score based on predicted pathogenicity. B) Tool that used

the immune response variant score derived from HIRS analysis estimates and hemophilia genotype score based on predicted impact on gene function. C) Tool that used the immune response variant score derived from HIRS analysis estimates and hemophilia genotype score based on previously-published evidence of effect of variant category.

ROC: receiver operating characteristic

X Val: cross-validation

Chapter 7: Summary, strengths, limitations, public health implications, and future research

Summary

Inhibitors present the most challenging treatment-related complication among persons with HA.[128] Understanding which persons with HA are at highest risk of inhibitor development is important, as treatment could be altered to lower risk. The only validated inhibitor risk prediction tool[7] has several limitations[8], including its reliance of information about family history and prior treatment. Furthermore, it has been validated only among persons with severe HA. A tool that relies on information that could be available at HA diagnosis may prove more useful clinically. This dissertation explores the development of a risk prediction tool based on genetic characteristics.

Two types of genetic risk factors for inhibitor development have been extensively studied: the HA genotype and variation in genes in the immune response pathway. Hemophilia-causing variants predicted to produce no gene product present the greatest risk, as any exogenous FVIII would be seen as foreign by the immune system.[10] Furthermore, variation in the immune response pathway that could alter function of genes important in immune response could also influence inhibitor risk.[52]

In **Aim 1** (Chapter 3) the ability to predict inhibitor status using information about the hemophilia genotype was explored. Three different tools to categorize the hemophilia genotype were assessed. None of the tools performed better than the previously-validated risk prediction tool. The best-performing tool assigned a score to hemophilia genotypes using severity-specific estimates of effect generated from previous investigations.

In **Aim 2** the relationship between variation in genes in the immune response pathway and inhibitors was explored. Numerous studies have investigated the relationship between variation in genes in the immune response pathway and inhibitors among persons with HA. However, the

degree of consistency of results and methods used among these studies had not been formally examined. A systematic review and meta-analysis was conducted (Chapter 4) in order to summarize previously-published results and to identify variants that were consistently associated with inhibitors. Summary estimates of effect were calculated for 94 genetic variants in 13 genes. Two variants in Class II HLA genes (*HLA-DQB1**0602 and *HLA-DRB1**15) and one variant in *TNFA* (rs1800629) were associated with increased prevalence of inhibitors. Two variants in IL10 were associated with decreased prevalence of inhibitors (rs1800896 and microsatellite repeat rs2234662). The examination of consistency of methods an results revealed a general lack of consistency in methods used and variants investigated. An additional analysis was also conducted to identify novel loci that may influence inhibitor risk (Chapter 5). Variants in *HLA-B*, *HLA-DPB1*, *IL1A*, *IL12B*, *CD80*, and *IL10* were found to be associated with inhibitor prevalence among persons with HA enrolled in HIRS.

In **Aim 3** (Chapter 6) the ability to predict inhibitor status based on information about the hemophilia genotype and variation in genes in the immune response pathway was evaluated. Results from **Aim 1** and **Aim 2** were combined. A total of six tools were evaluated. While none of the tools evaluated performed better than the previously-validated tool in which the AUC was 0.74[7] among persons with severe disease, one tool performed well among those with severe disease (AUC=0.65) and better than the previously-validated tool among persons with mild/moderate disease (AUC=0.80), without requiring treatment or family history information. This tool combined information about the hemophilia genotype categorized based on previously-generated estimates of effect along with genotype information about loci in *HLA-DPB1*, *IL1A*, *IL12B*, *CD80*, and *IL10*.

Although none of the tools evaluated performed better than the previously-validated tool[7] among persons with severe disease, among whom the risk of inhibitor development is highest, the ability of utilizing genetic information to predict inhibitor status shows promise. While the

AUC for the previously-validated tool[7] was reported to be 0.74, subsequent validation studies[126,164] conducted in other populations indicated suboptimal performance (AUC 0.65-0.71). This indicates that the best-performing tool that utilizes information on a limited set of genetic predictors may perform similarly to a tool that requires information that is not readily available for a large subset of persons with hemophilia. This highlights the potential utility of a genetics-based inhibitor prediction tool.

Strengths

Considering hemophilia is a rare disease, this investigation utilized data from a relatively large, racially and ethnically homogeneous sample of persons with HA in the United States, helping to control for the confounding effect of race on genetic associations in this population. HIRS included nearly 7% of the entire US population of persons with HA. The large sample size allowed the evaluation of a wide range of genetic risk factors for inhibitors. While the determination of inhibitor status was not based on testing by a central laboratory in most cases, all inhibitor-positive enrollees had a documented inhibitor titer of at least 1 Bethesda Unit. Miller et al report false positive inhibitor measurements are most likely in the 0.5-1.0 BU range.[202] This makes misclassification of the outcome unlikely. Furthermore, many had documentation of use of inhibitor treatment or use of bypassing agents.

The systematic review and meta-analysis conducted as part of this dissertation is the largest, most comprehensive review of the literature regarding associations between variants in genes in the immune response pathway and inhibitors among persons with HA. When data were not published in a way that would allow computation of summary estimates of effect or if data were referenced but unpublished, authors were contacted in order to standardize results and be comprehensive.

Where possible, external data were used to provide estimates for the genetic risk prediction model. When estimates derived from HIRS-based analyses were used, cross-validation methods

were used to reduce the bias introduced by using the same data to simultaneously fit and assess the performance of the model.

Limitations

While HIRS is a relatively large study of HA in the United States, the sample size limited the power to detect novel genetic associations. Even though permutation procedures were used to identify novel associations in the analysis of variants in genes in the immune response pathway instead of the more conservative Bonferroni multiple-testing correction, a larger sample size would be needed to detect variants that could introduce small changes in inhibitor risk or variants that would introduce large changes in inhibitor risk but are rare in the population. Furthermore, while the use of a racially and ethnically homogeneous sample of persons with HA reduced the impact of confounding, this also limits the generalizability of the results. In the best-performing inhibitor prediction tool, information on variants in *HLA-DPB1*, *IL1A*, *IL12B*, *CD80*, and *IL10* identified by associations with inhibitors in this sample was included as part of the model. The allelic distribution of these variants varies by race/ethnicity[203], making it unlikely that this model would perform in the exact same manner in other race/ethnicities. Validation studies of the model using other patient populations are needed to determine how well the model will perform as a prediction tool.

Public Health Implications

The results of this investigation indicate an inhibitor risk prediction tool based on genetic information shows promise. The relatively simple tools presented in this dissertation include a limited set of genetic predictors that could be available at the time of hemophilia diagnosis. A tool that relies on information that could be available at the time of hemophilia diagnosis could be more useful clinically when deciding treatment strategy. Because the treatment strategy could be altered depending on genetic predictors, the tool would likely be considered precision medicine; however, the potential reduction in occurrence of inhibitors among persons with HA could have public health implications. Inhibitors represent the greatest treatment-related

complication among persons with HA, causing increased bleeding[3] and mortality risk[4,25] and healthcare costs[3,5,34-40]. The ability to understand which persons with HA may be most likely to develop inhibitors and implementation of treatment changes that may alter risk could reduce inhibitor occurrence, thereby improving outcomes among persons with HA.

Future Research

This investigation was conducted among White, Non-Hispanic persons with HA enrolled in HIRS and were not validated in an external population. Future efforts should aim to validate these results.

The My Life, Our Future project[69] offers an opportunity to validate the results of this investigation. The My Life, Our Future Research Repository contains information from nearly 10,000 persons with hemophilia and carriers of hemophilia enrolled from more than 100 participating hemophilia treatment centers. The hemophilia genotype of each participant was determined. Whole genome sequencing was also carried out for nearly 5,000 enrollees. Clinical data, including inhibitor phenotype, is available for each participant. A research proposal is being developed that would utilize the My Life, Our Future Research Repository data to validate the performance of the inhibitor risk prediction tools developed in this investigation and to identify novel genetic loci that may influence inhibitor risk, the information about which could be added to future iterations of inhibitor risk prediction tools to improve performance.

The population sample for this investigation was limited to White, Non-Hispanic persons with HA in order to limit the potential confounding impact of race/ethnicity. However, this limits the generalizability of the results of this investigation. As the allelic distribution of many of the variants included in the inhibitor risk prediction tools are expected to vary by race/ethnicity[203], the performance of the tools in other populations is questionable. HIRS was conducted in several phases. During the first two phases, low enrollment of minority populations was identified as a limitation. HIRS Phase III aimed to enroll more minority populations, in order to

investigate inhibitor risk factors in these populations. An additional 136 Black and 120 Hispanic persons with HA were enrolled. Enrollment for HIRS Phase III ended in April 2016. Hemophilia genotyping was completed for enrollees in June 2017. Efforts to genotype variants in genes in the immune response pathway are currently underway. Once these data are available, the performance of the inhibitor risk prediction tools presented in this investigation can be evaluated. Furthermore, these data could be used to identify novel loci that may be particularly important in affecting inhibitor risk in minority populations.

Finally, understanding which persons with HA may be most likely to develop inhibitors is important, but even more important is understanding what to do with that information. It has been suggested that adjusting treatment strategies to avoid intensive exposure[62-64] or exposure to recombinant products[65] during the first few exposure days may reduce risk, particularly among those at highest risk of inhibitor development. However, the actual impact of these treatment changes remains to be determined. Future efforts should aim to evaluate the efficacy and effectiveness of genetics-guided treatment strategies to reduce inhibitor risk among persons with HA.

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Supplementary Information

Chapter 3

ACMG Standards and Guidelines for Interpretation of Sequence Variants

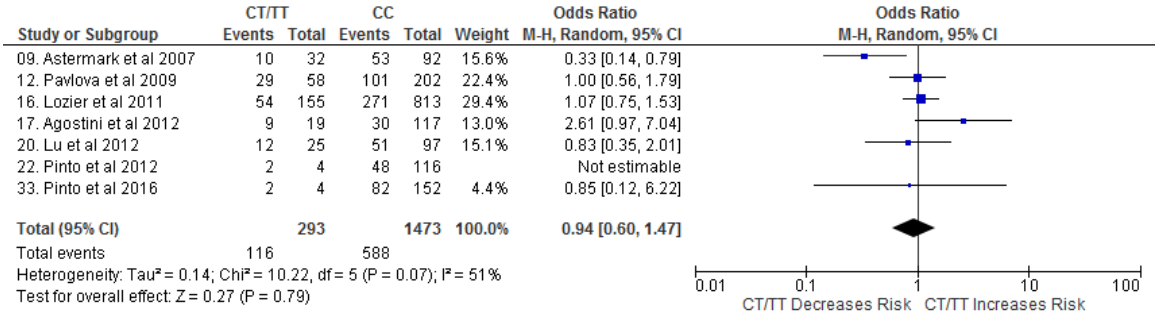
Comments	Pathogenic					Benign			Comments	Dissertation Operationalization
	Very Strong (4pts)	Strong (3pts)	Moderate (2pts)	Supporting (1pt)		Stand-alone (-3 pt)	Strong (-2pt)	Supporting (-1pt)		
NS, FS, ±1 or 2 splice sites, Met, exon deletion => LOF known MOD	PVS1				Null Variants					Classified any nonsense, frameshift, ± 1 or 2 splice site, inversion, large duplication (>= 1 exon), large deletion (>=1 exon) variant as PVS1
Same codon, dif nt		PS1			Same AA change					Searched CHAMP for variants that had same AA change but different nucelotide change. NOTE: none of the duplicate pairs were both from HIRS.
in vitro or *in vivo* study		PS3			Functional studies		BS3		*In vitro/in vivo* study shows no damaging effect	Searched PubMED for references of functional studies.
PM2: Not in controls (EVS, 1000G, ExAC).			PM2		Variant frequency and use of control populations	BA1	BS1, BS2		BA1: MAF >5% EVS, 1000G, ExAC. BS1: MAF greater than expected. BS2: Healthy indiv. as Homo(AR), Het(AD), Hemi(XL) at full pen.	Annotated MAF in EVS, 1000G, ExAc using ANNOVAR.
No benign variants in hotspot			PM1		Mutational hotspot / critical and well-established functional domain					Non-truncating: Outside B domain Truncating: Anywhere in gene.
In-frame indel in nonrepeat region or stop-loss variants			PM4		Protein length changes due to in-frame indels and stop losses			BP3	In-frame indel in repetitive region without known function	In-frame indel outside B domain.
Different missense at same codon determined to be pathogenic			PM5		Novel missense at the same position					Searched CHAMP for variants that had AA change at codon previously associated with disease. NOTE: excluded variants only reported in HIRS.
Coseg with multiple affected family members. Gene definitively known to cause disease				PP1	Segregation analysis		BS4		Lack of seg in affected family members	Gene definitively known to cause disease.
Missense in gene with low benign missense variation, missense common MOD.				PP2	Variant spectrum			BP1	Missense when primarily truncation variants	Missense common MOD.
Multiple data support deleterious effect on gene/product				PP3	Computational (*in silico*) data			BP4	Multiple data shows no impact on gene/product	CADD, SIFT, PolyPhen2 scores annotated via ANNOVAR. At least 2 programs predicted deleterious.
Patient's pheno or family Hx highly specific for disease with single cause			PM6		Using phenotype to support variant claims					Highly specific phenotype.
Reputable source recently reports variant as pathogenic (no evidence)				PP5	Reputable source			BP6	Reputable source recently reports variant as benign (no evidence available)	Reported in CHAMP (not in HIRS).
					Synonymous variants			BP7	Silent variant with no predicted impact on splicing; lack of conservation	NOTE: all synonymous variants shown to be deleterious by functional studies

Supplementary Table 1: Operationalization of Pathogenicity Tool.

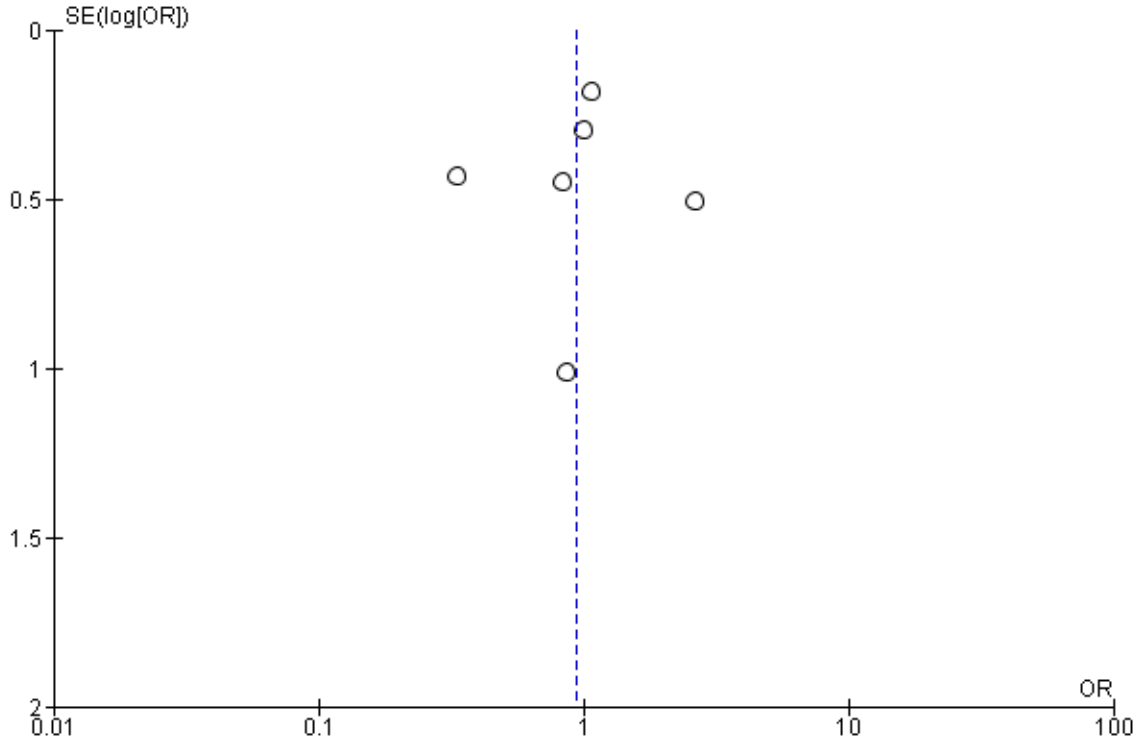
Chapter 4

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R
1	Publication Info			Population Info						Study Design				Results				
2	Author	Year	Study Name	Race/Ethnicity	Severity	Total N	Inhibitor Positive N	Inhibitor Negative N		Inhibitor Positive Definition	Inhibitor Negative Definition	Dates Data Collection	Study Type	Variant Tested	Variant Reported	REF/REF N	REF/ALT N	ALT/ALT N
3																		
4																		
5																		
6																		
7																		
8																		
9																		
10																		
11																		

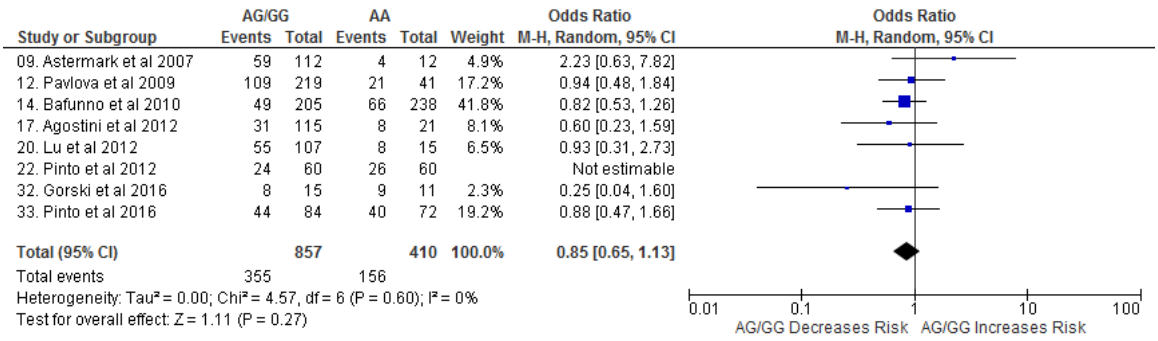
Supplementary Figure 1: Data extraction form used to collect information from reports included in systematic review



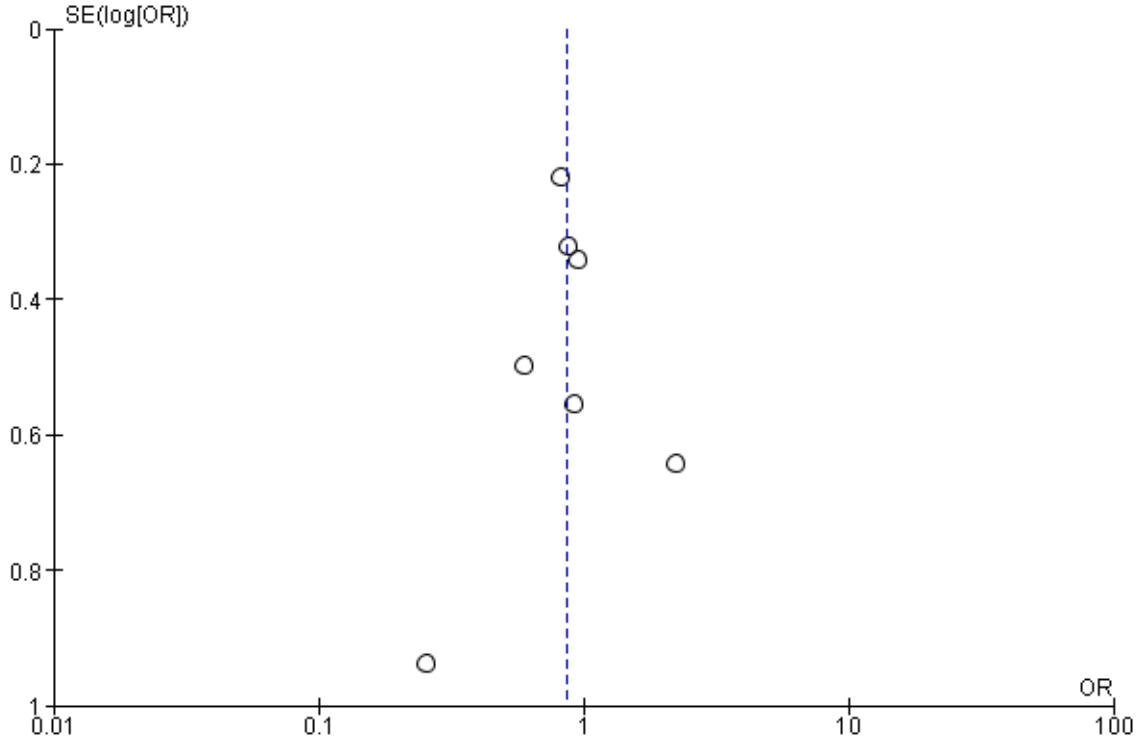
Supplementary Figure 2: Forest plot for *CTLA4* -318 C>T (rs5742909)



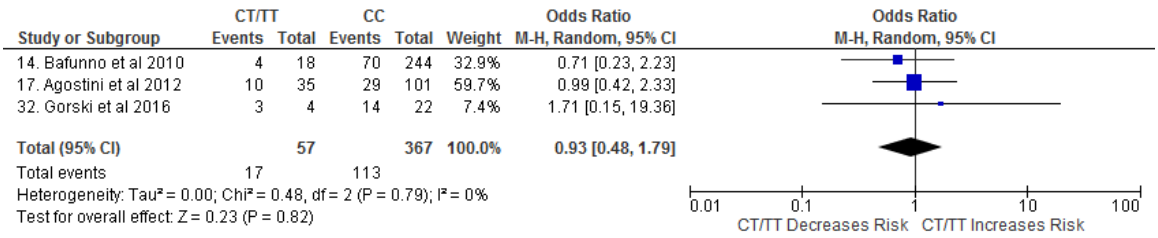
Supplementary Figure 3: Funnel plot for *CTLA4* -318 C>T (rs5742909)



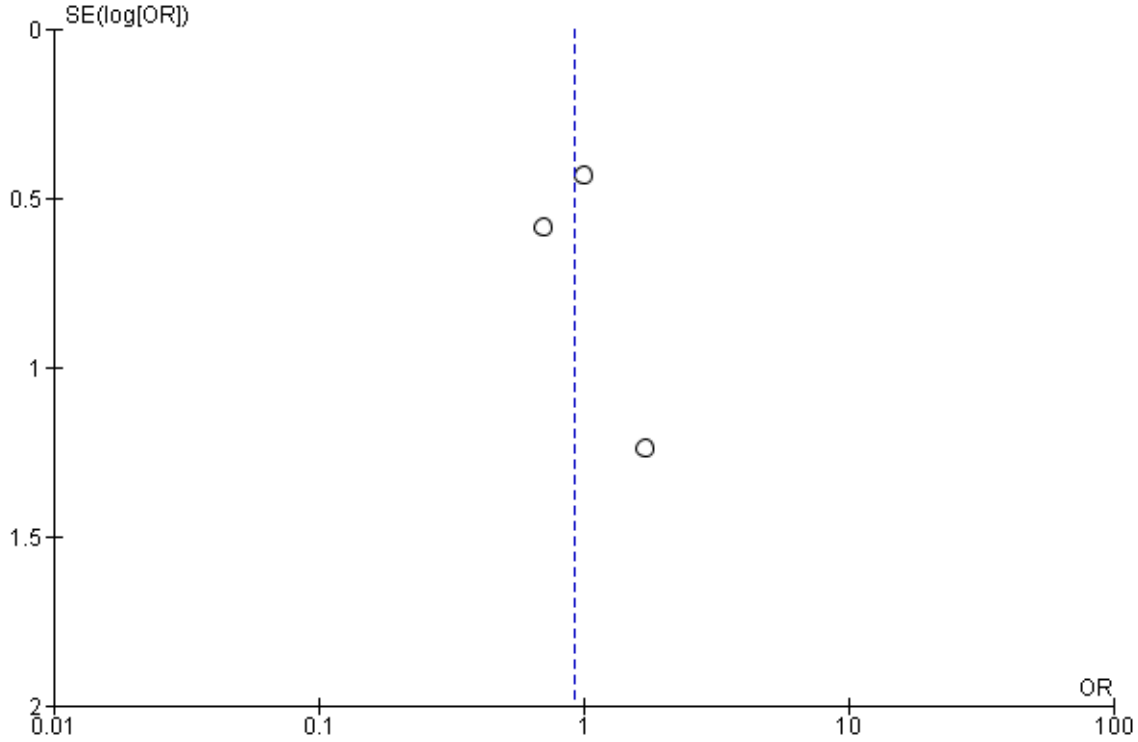
Supplementary Figure 4: Forest plot for *CTLA4* 49 A>G (rs231775)



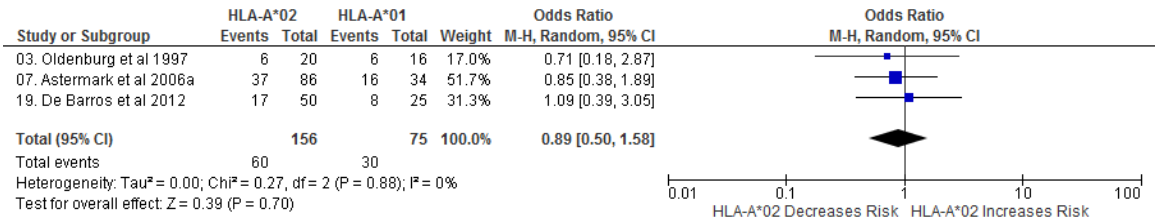
Supplementary Figure 5: Funnel plot for *CTLA4* 49 A>G (rs231775)



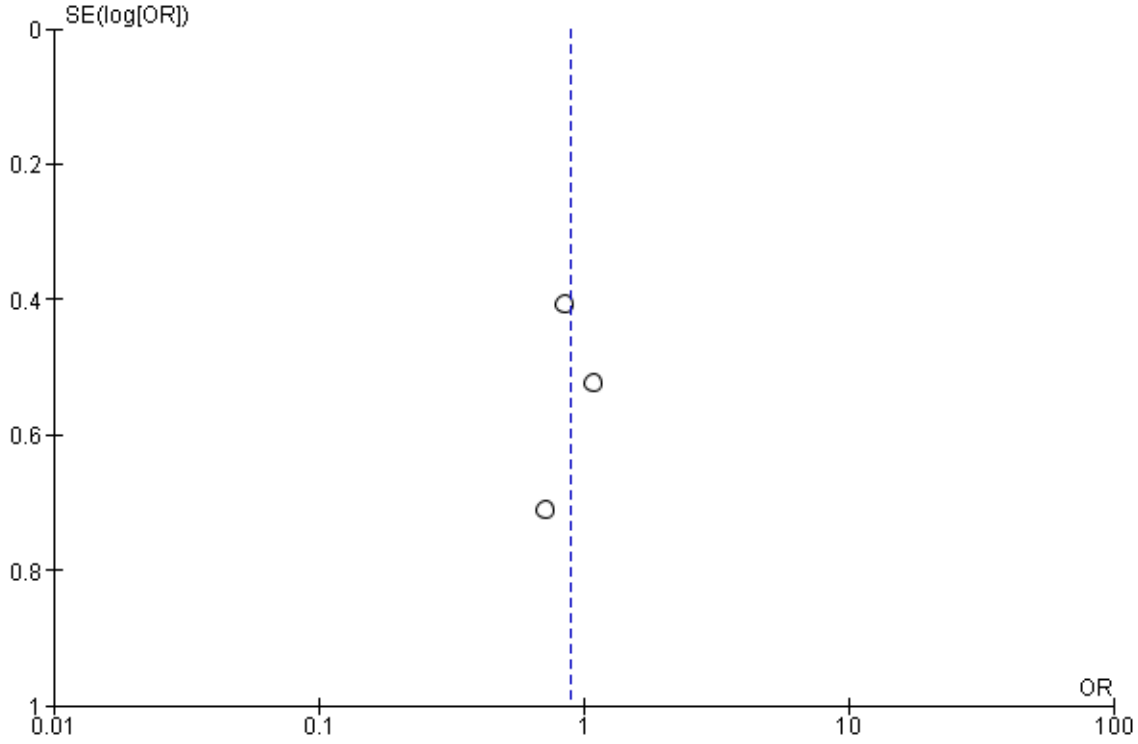
Supplementary Figure 6: Forest plot for *PTPN22* c.1858 C>T (rs2476601)



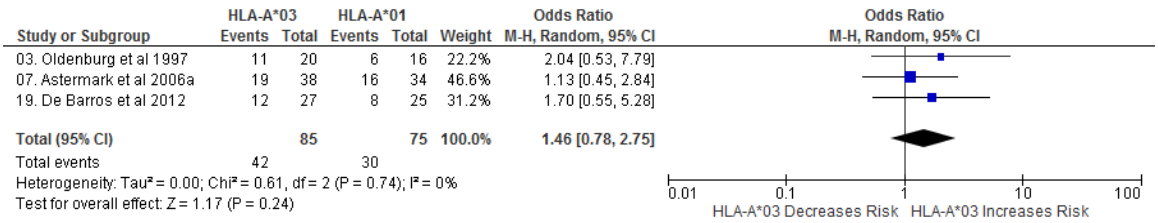
Supplementary Figure 7: Forest plot for *PTPN22* c.1858 C>T (rs2476601)



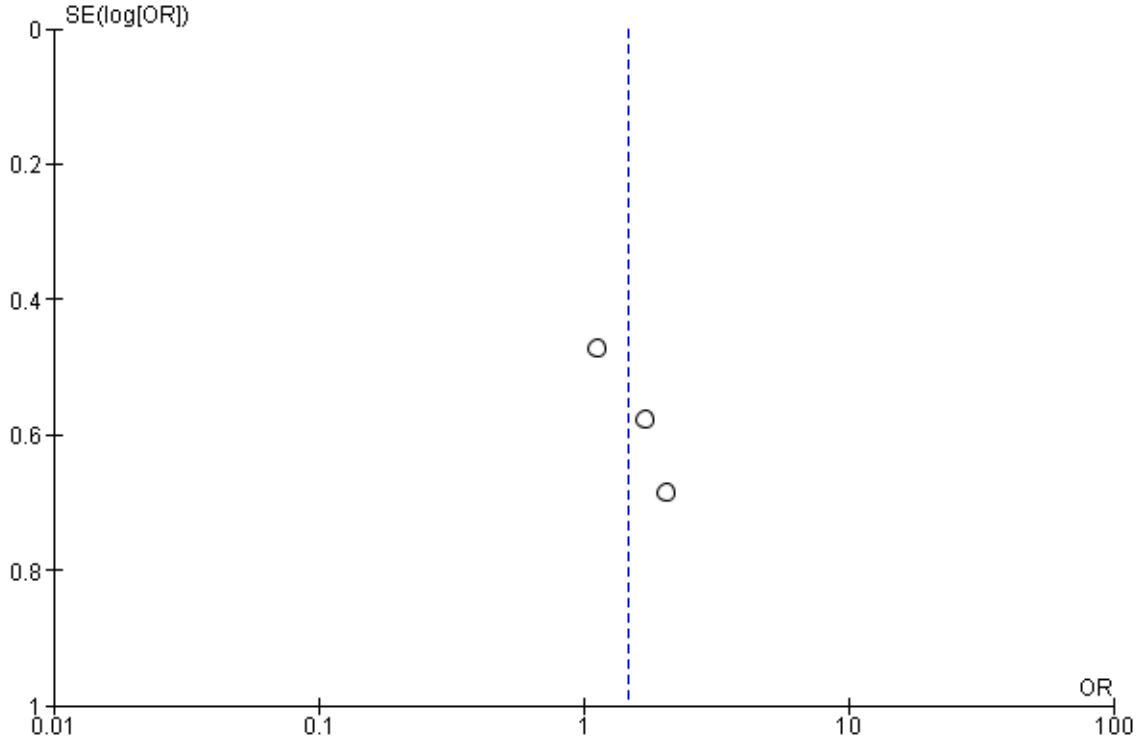
Supplementary Figure 8: Forest plot for *HLA-A02***



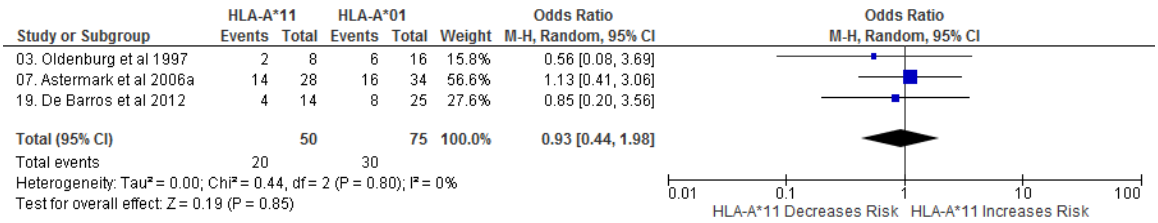
Supplementary Figure 9: Funnel plot for *HLA-A02**



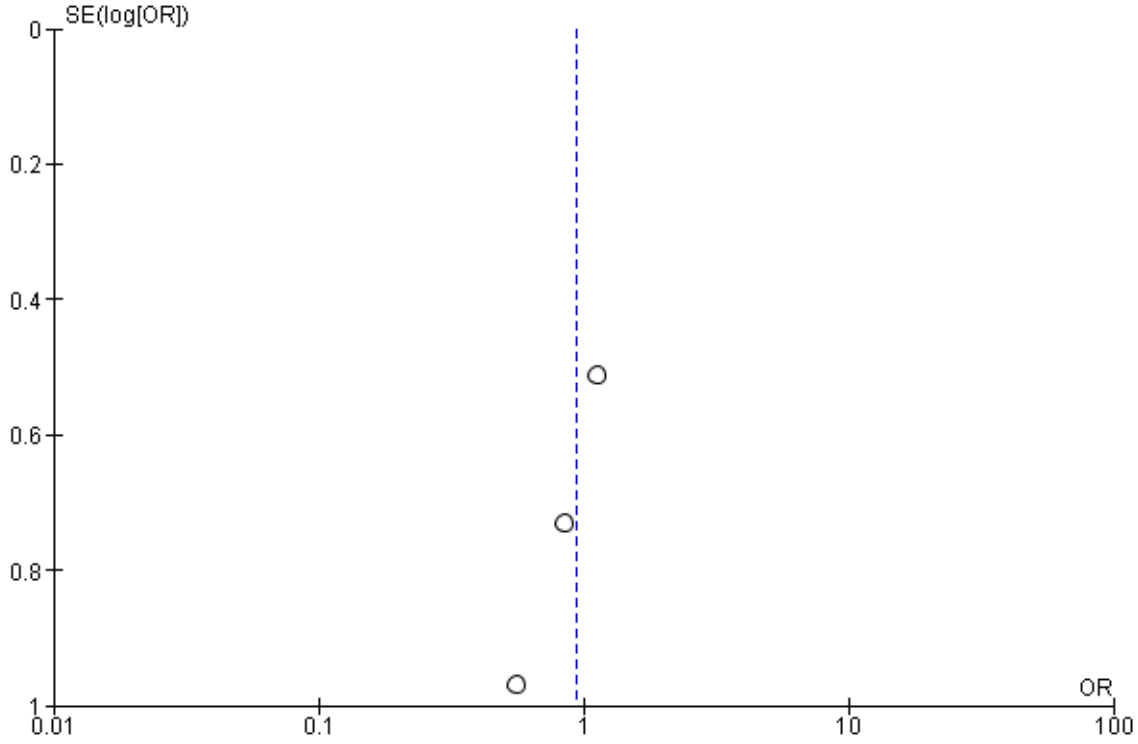
Supplementary Figure 10: Forest plot for *HLA-A03**



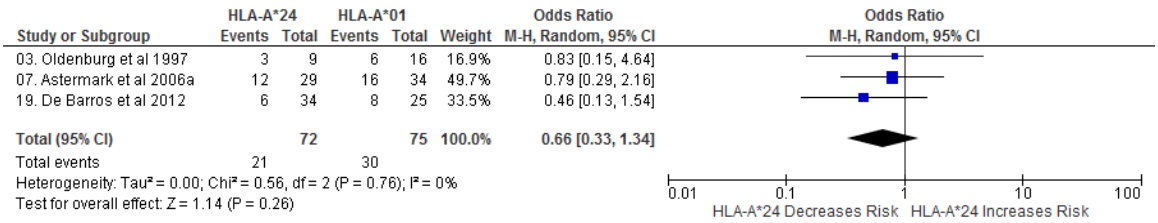
Supplementary Figure 11: Funnel plot for *HLA-A03**



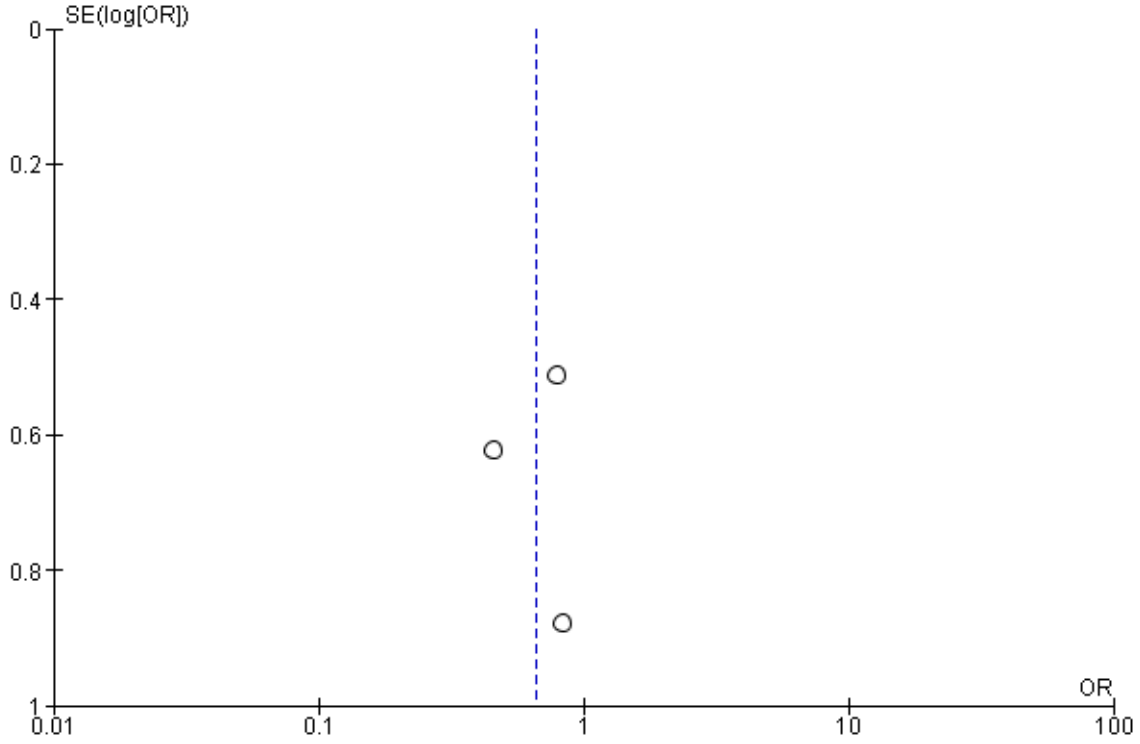
Supplementary Figure 12: Forest plot for *HLA-A11**



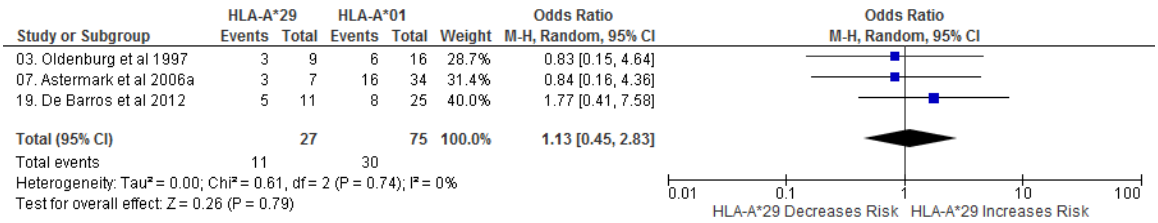
Supplementary Figure 13: Funnel plot for *HLA-A11**



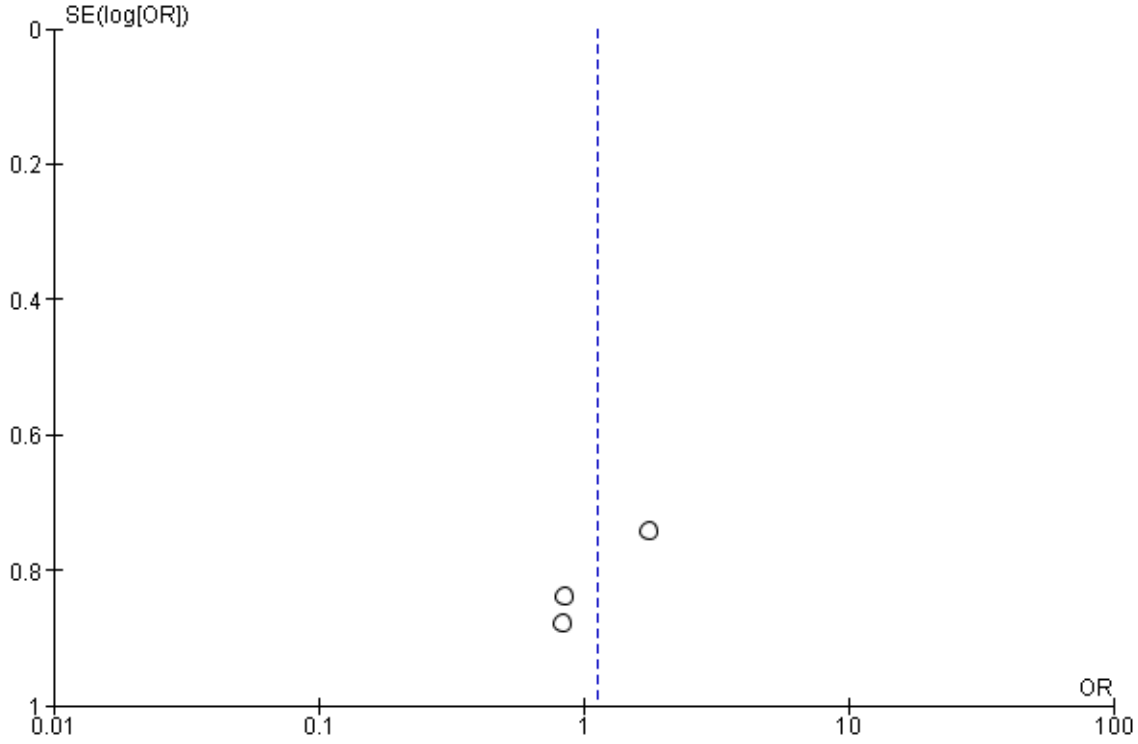
Supplementary Figure 14: Forest plot for *HLA-A24**



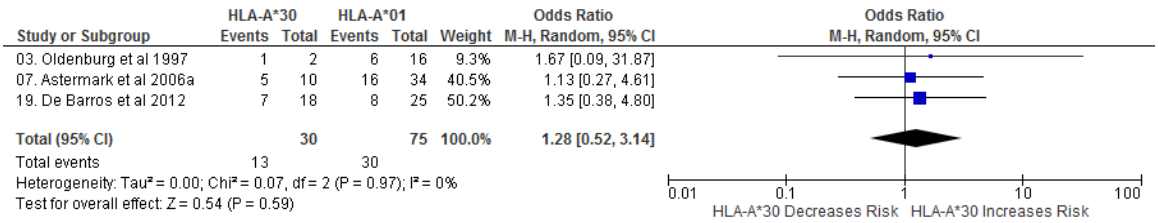
Supplementary Figure 15: Funnel plot for *HLA-A24**



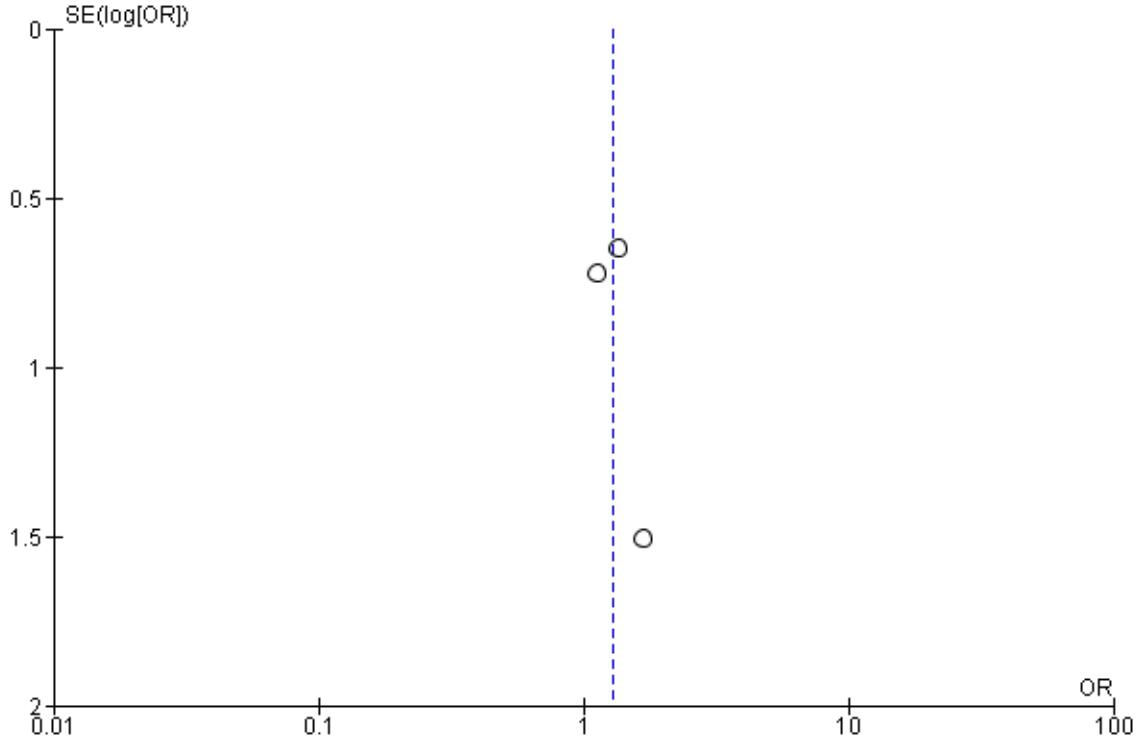
Supplementary Figure 16: Forest plot for *HLA-A29**



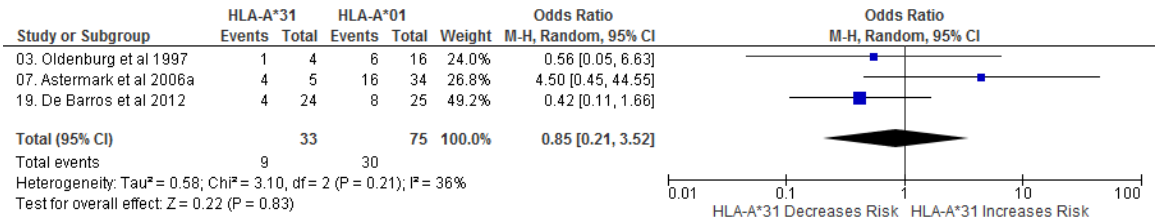
Supplementary Figure 17: Funnel plot for *HLA-A29**



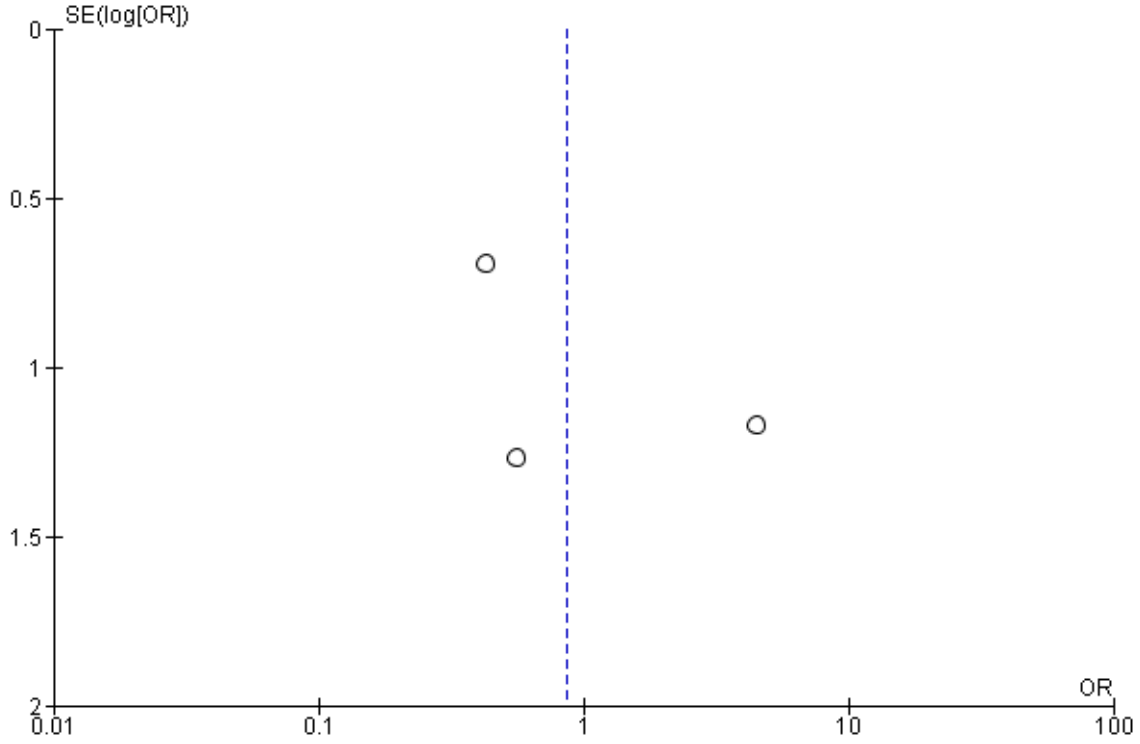
Supplementary Figure 18: Forest plot for *HLA-A30**



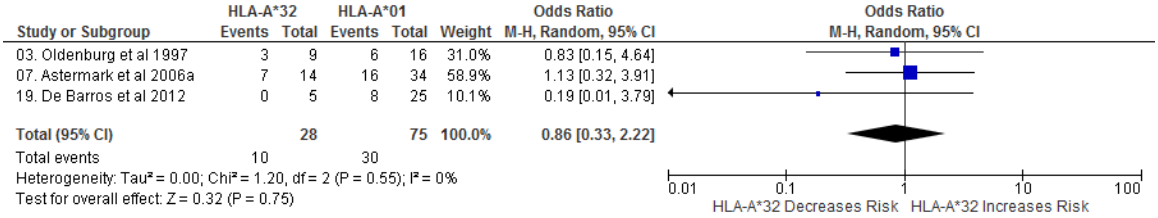
Supplementary Figure 19: Funnel plot for *HLA-A30**



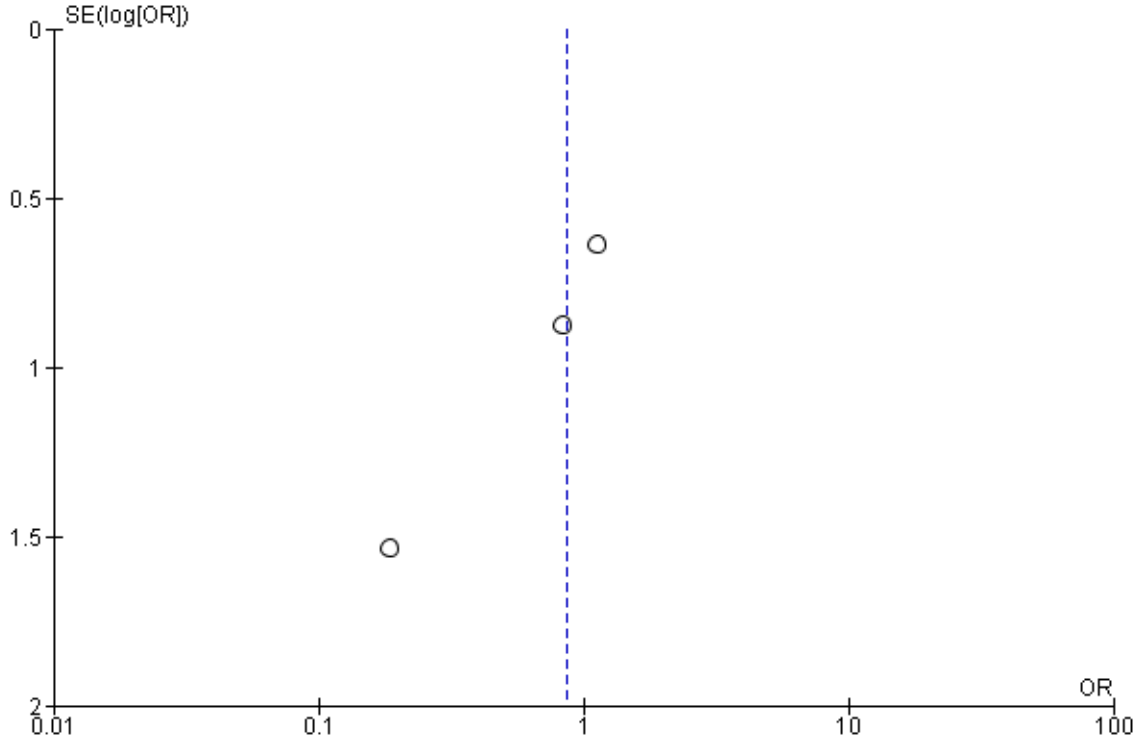
Supplementary Figure 20: Forest plot for *HLA-A31**



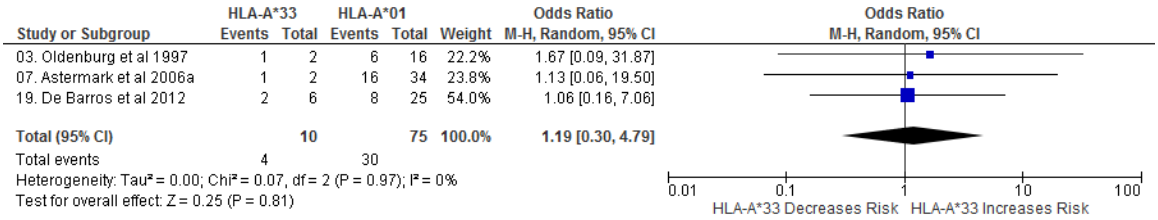
Supplementary Figure 21: Funnel plot for *HLA-A31**



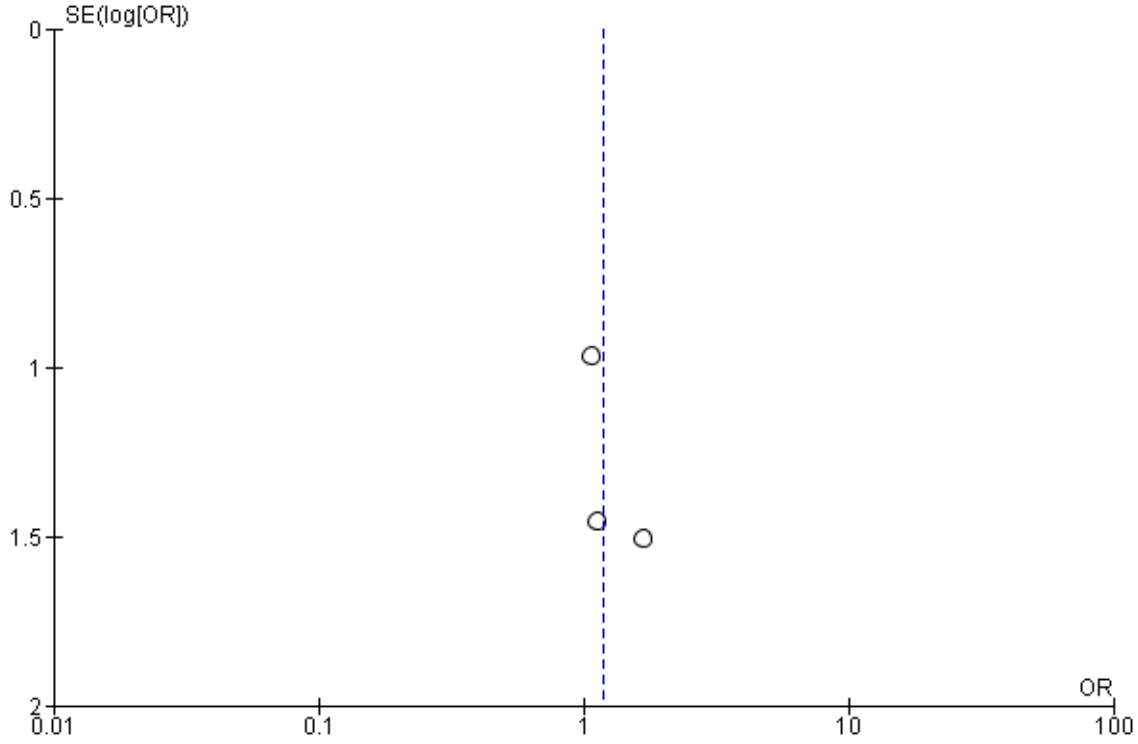
Supplementary Figure 22: Forest plot for *HLA-A32**



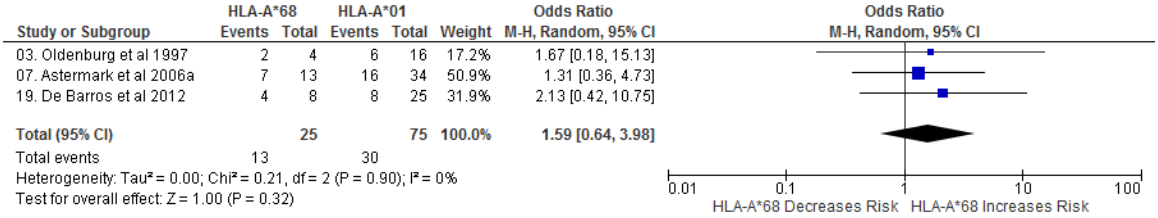
Supplementary Figure 23: Funnel plot for *HLA-A32**



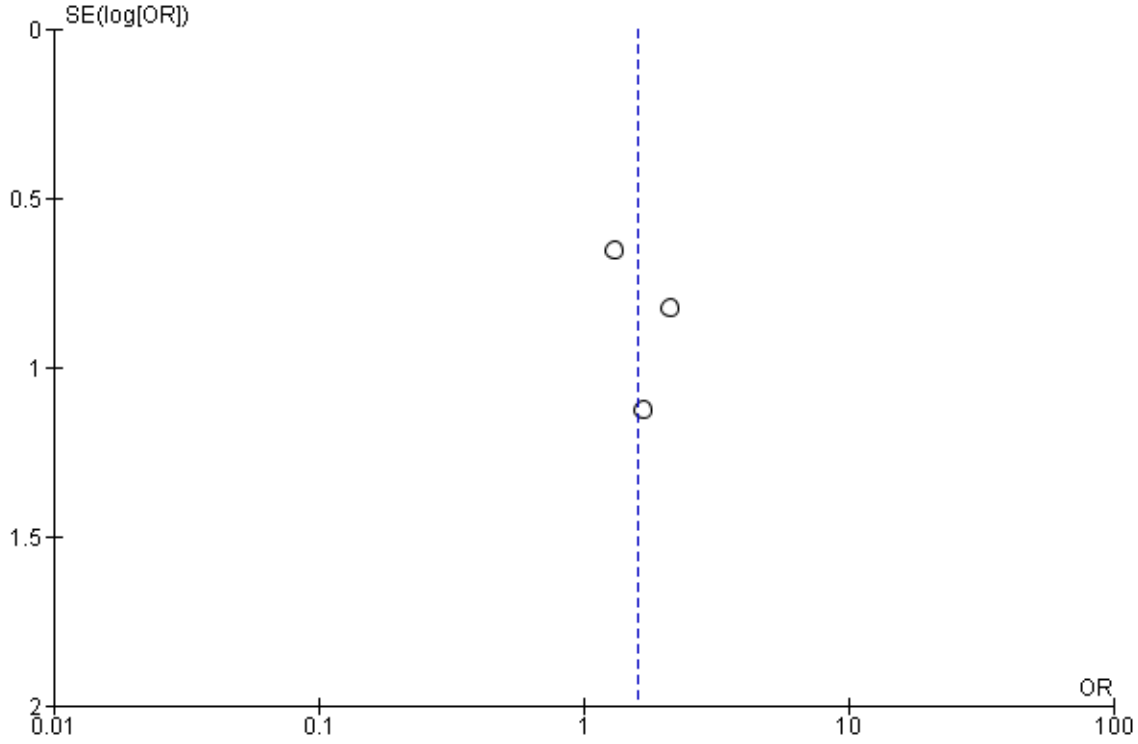
Supplementary Figure 24: Forest plot for *HLA-A33**



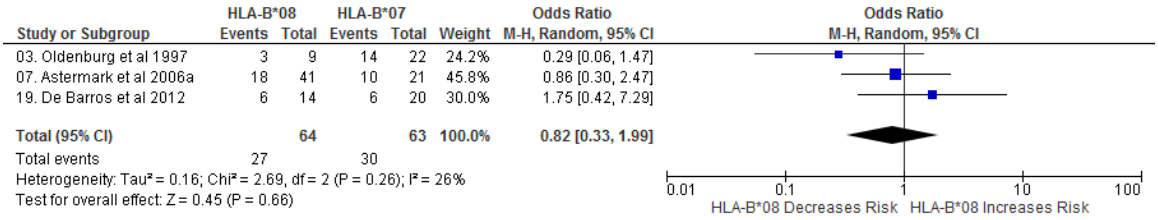
Supplementary Figure 25: Funnel plot for *HLA-A33**



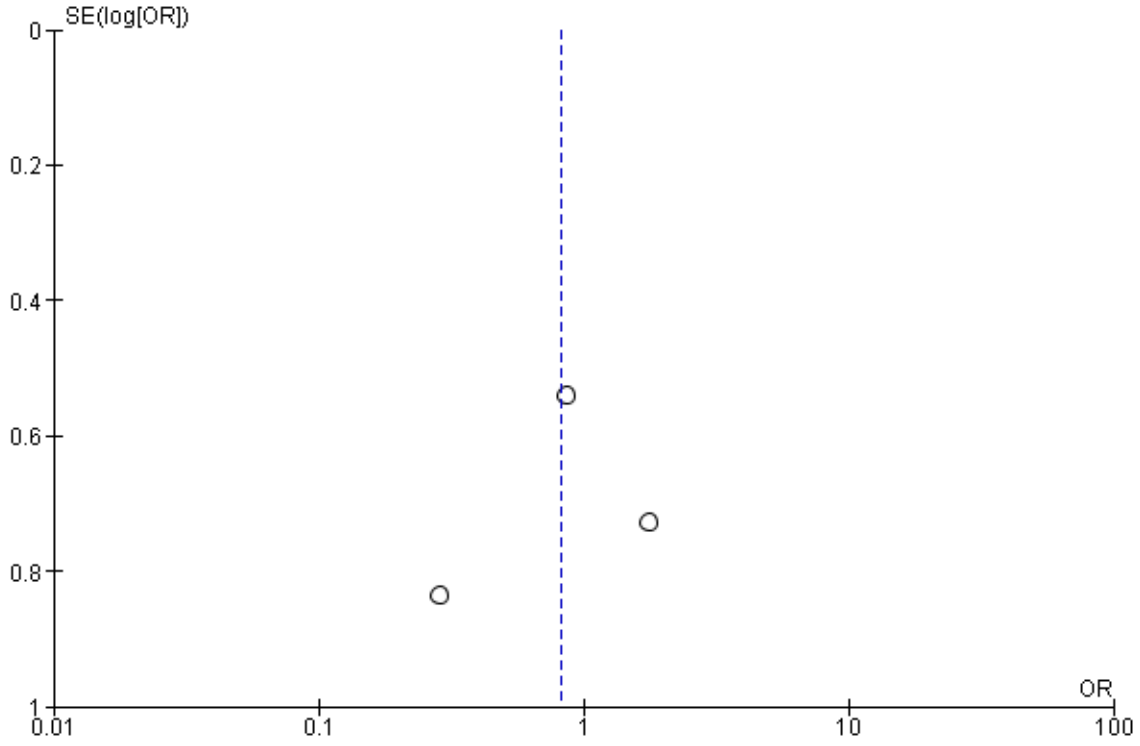
Supplementary Figure 26: Forest plot for *HLA-A68**



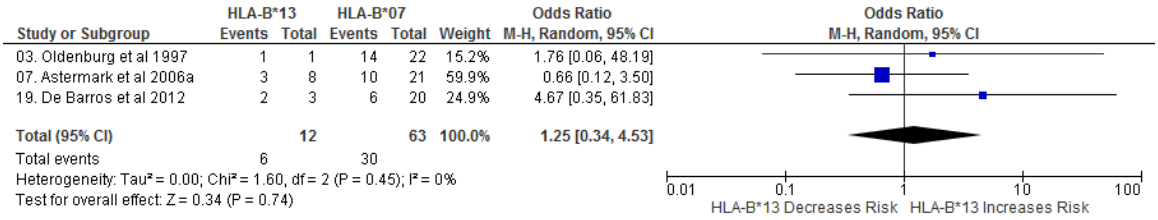
Supplementary Figure 27: Funnel plot for *HLA-A68**



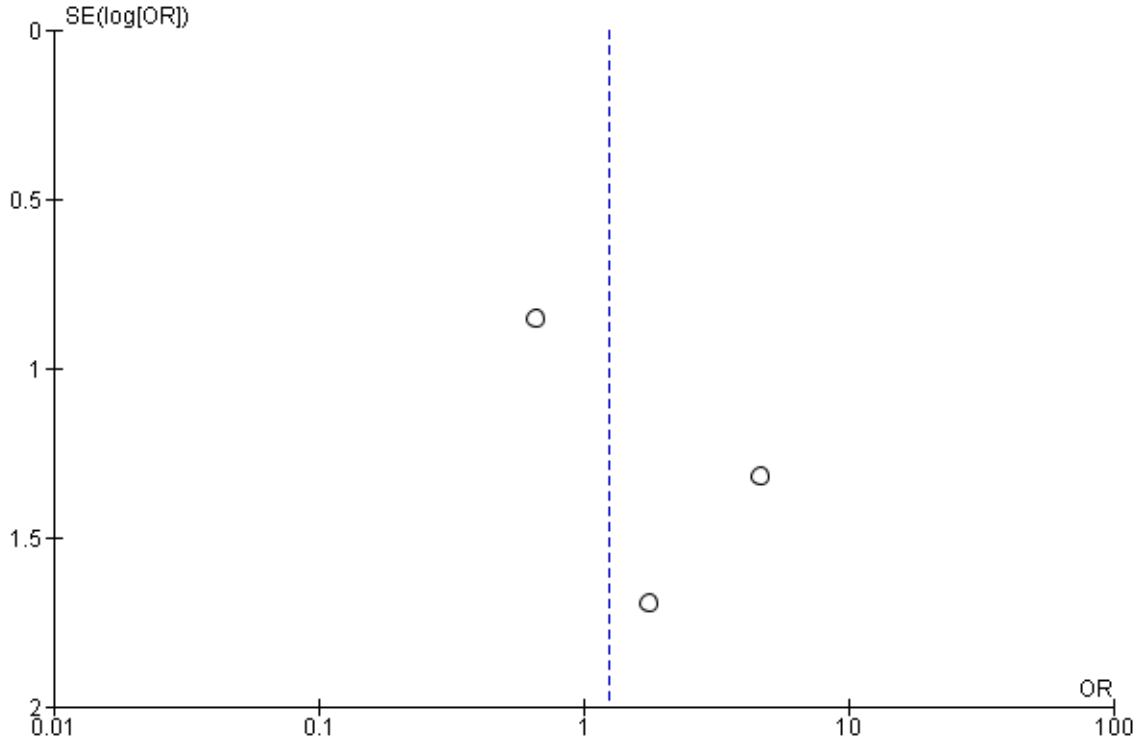
Supplementary Figure 28: Forest plot for *HLA-B08***



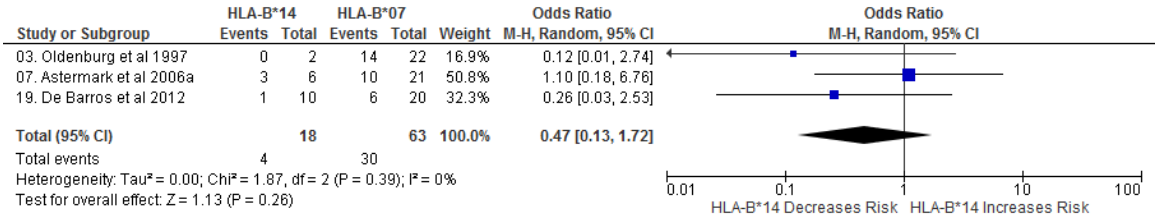
Supplementary Figure 29: Funnel plot for *HLA-B08***



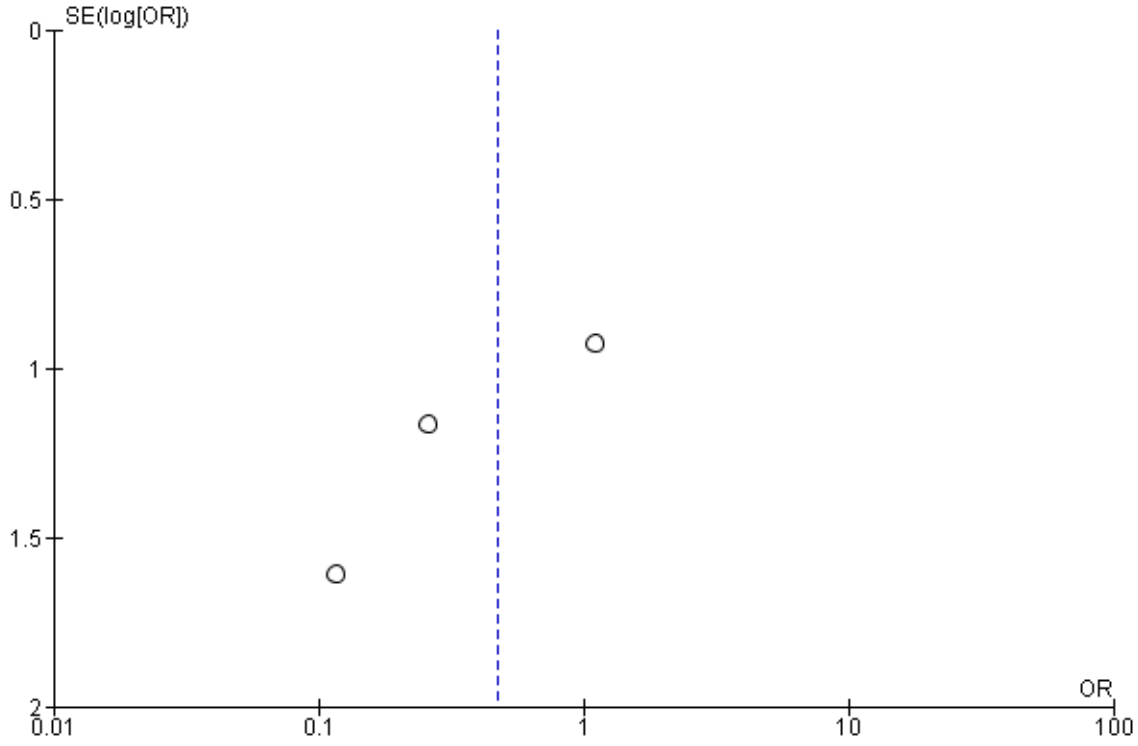
Supplementary Figure 30: Forest plot for *HLA-B13**



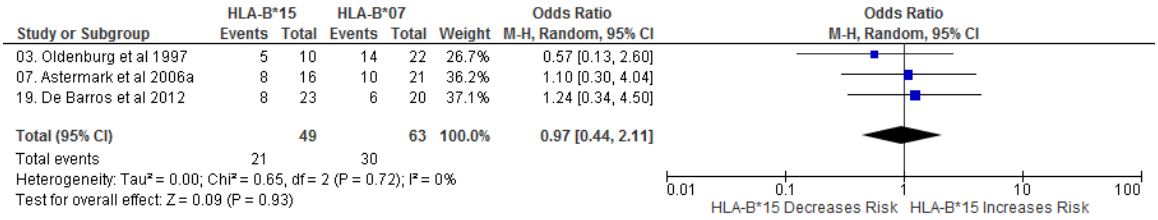
Supplementary Figure 31: Funnel plot for *HLA-B13**



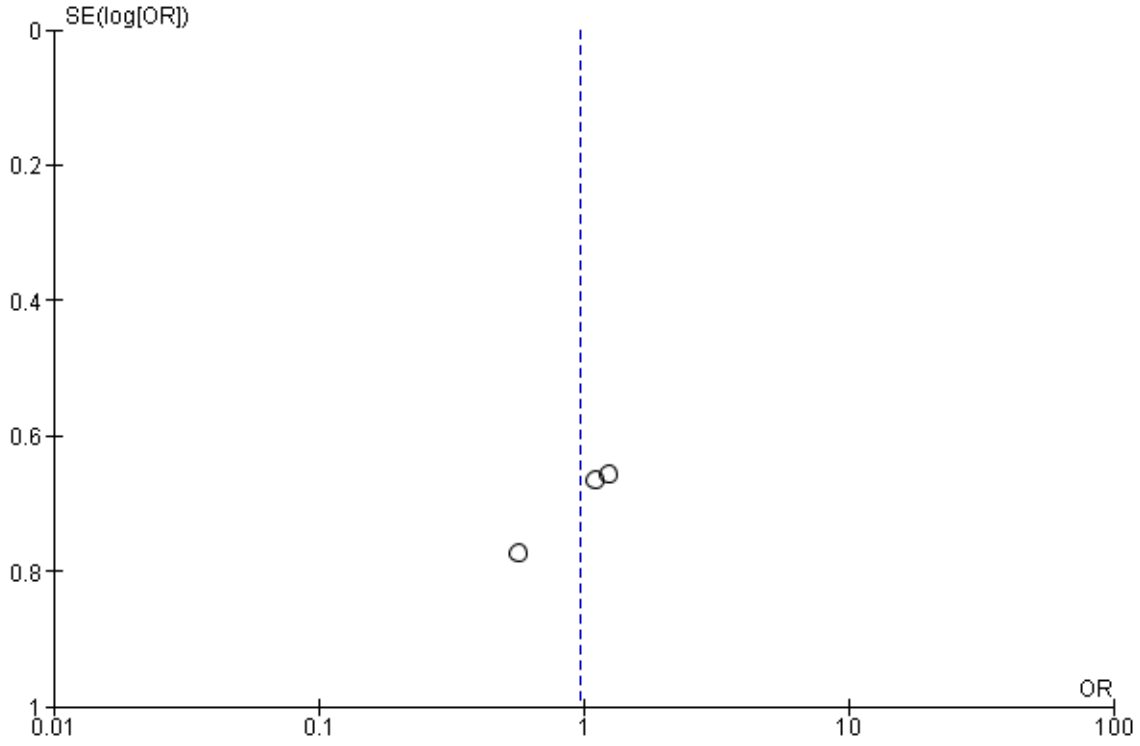
Supplementary Figure 32: Forest plot for *HLA-B14**



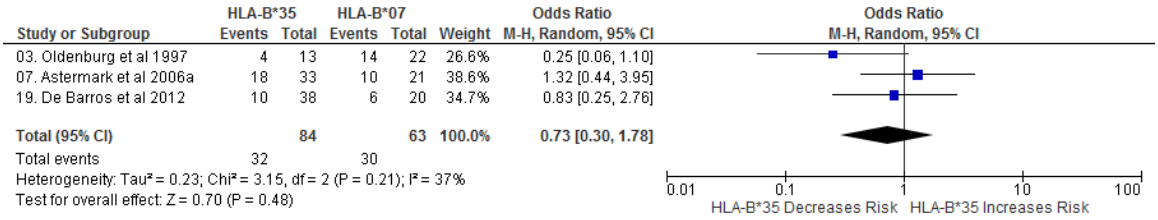
Supplementary Figure 33: Funnel plot for *HLA-B14**



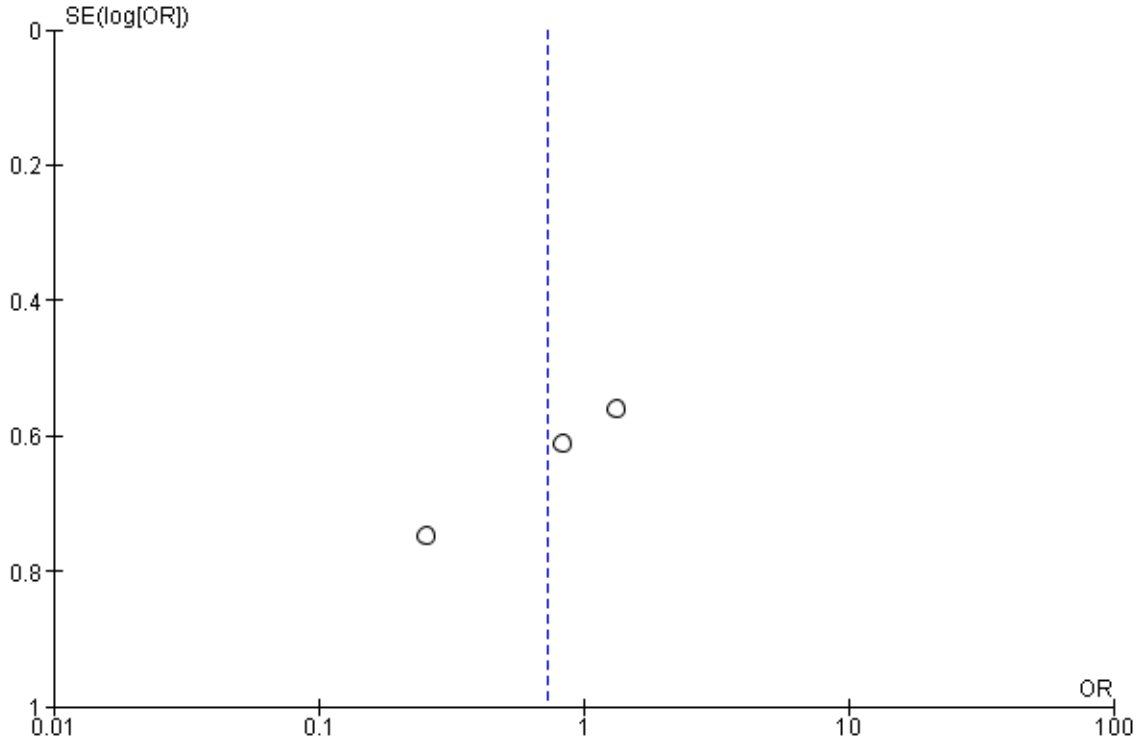
Supplementary Figure 34: Forest plot for *HLA-B15**



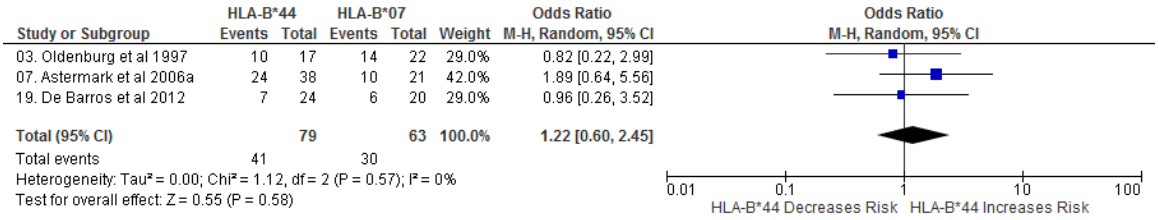
Supplementary Figure 35: Funnel plot for *HLA-B15**



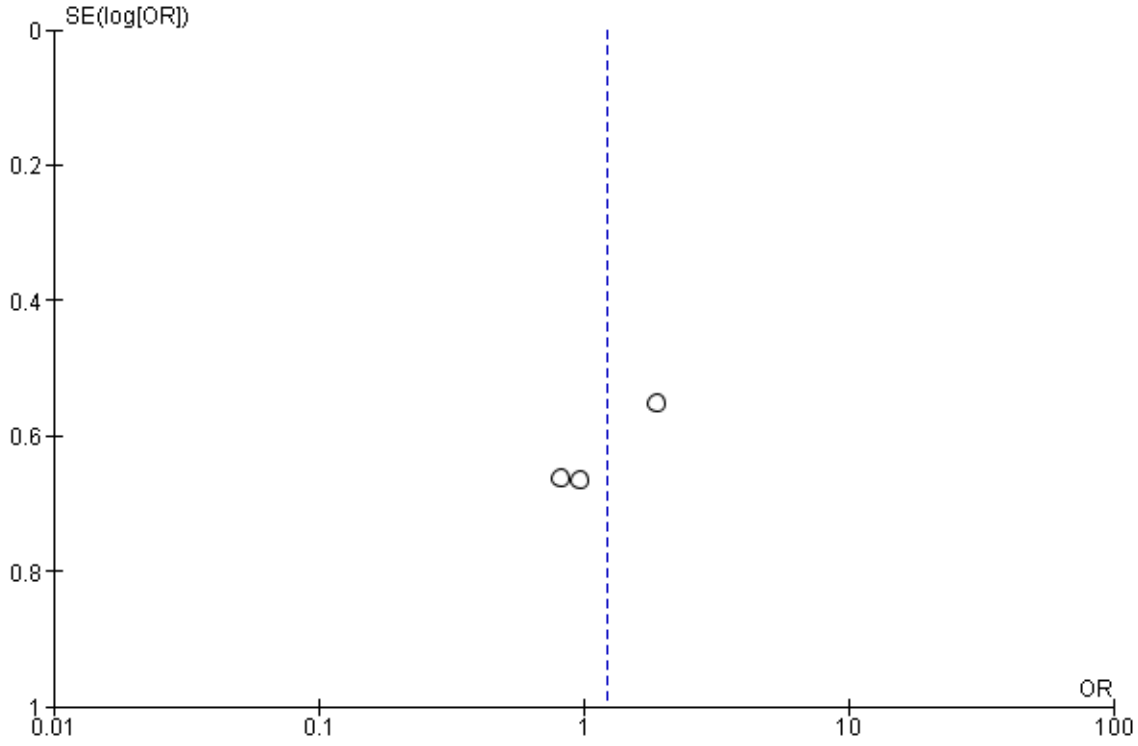
Supplementary Figure 36: Forest plot for *HLA-B35**



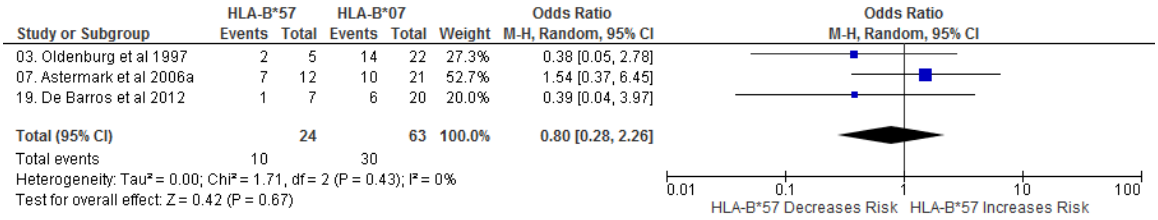
Supplementary Figure 37: Funnel plot for *HLA-B35**



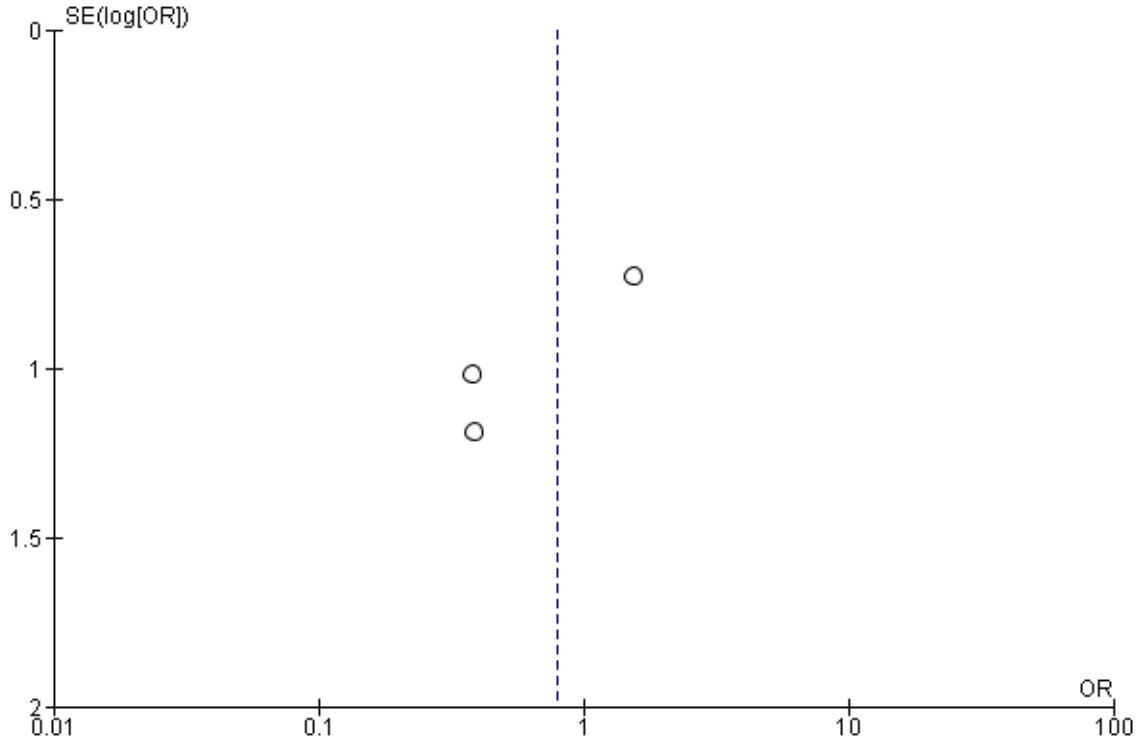
Supplementary Figure 38: Forest plot for *HLA-B44**



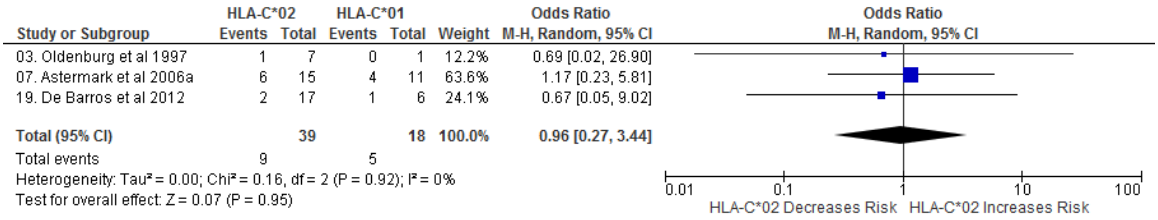
Supplementary Figure 39: Funnel plot for *HLA-B44**



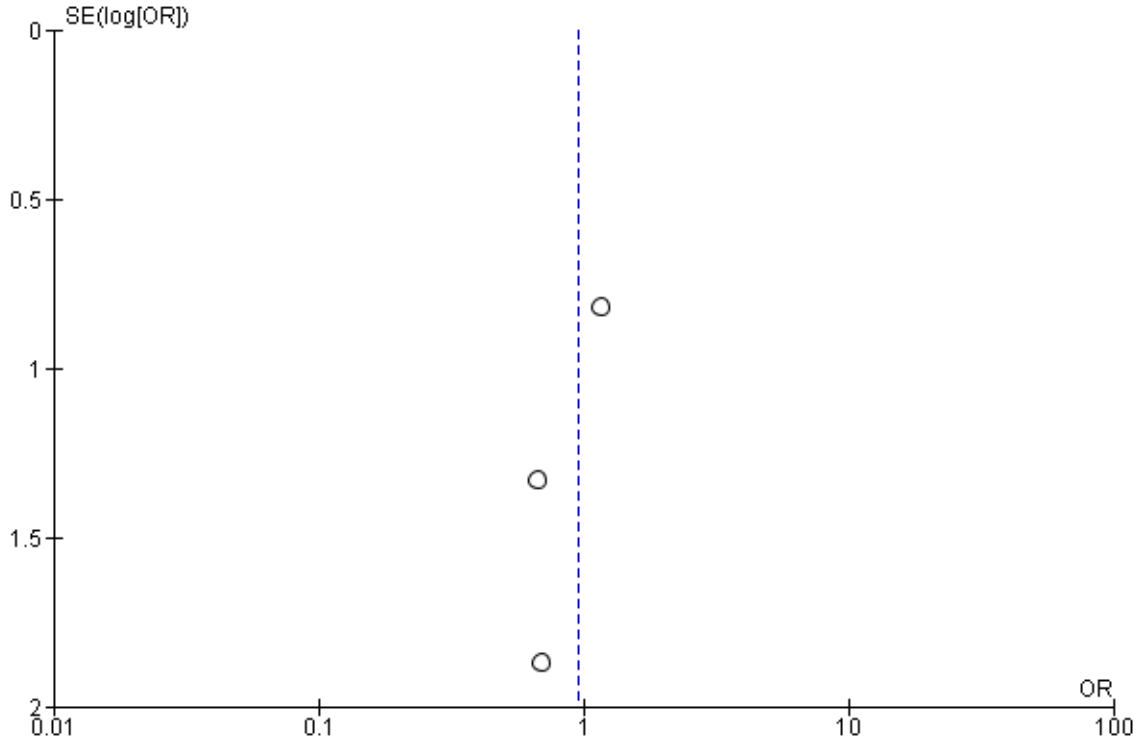
Supplementary Figure 40: Forest plot for *HLA-B57**



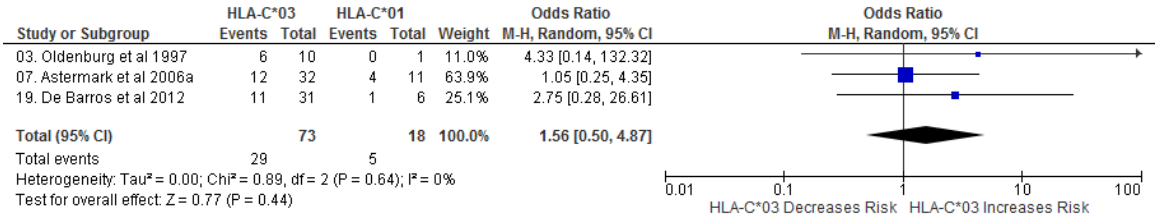
Supplementary Figure 41: Funnel plot for *HLA-B57**



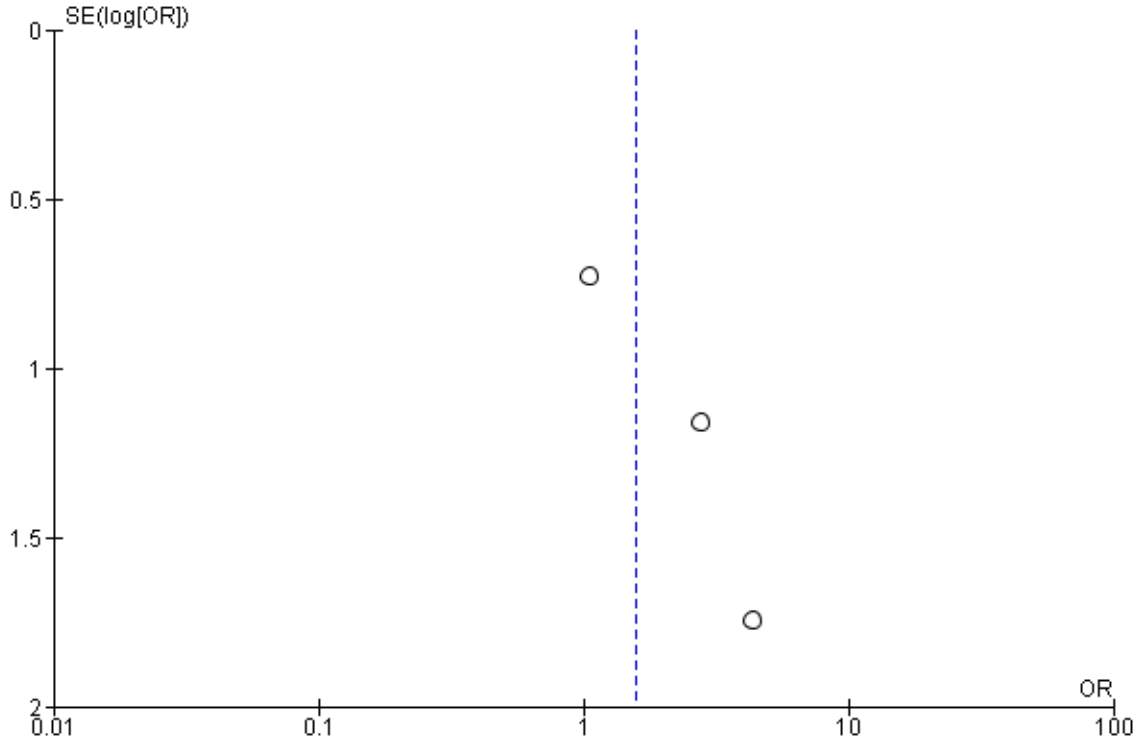
Supplementary Figure 42: Forest plot for *HLA-C02**



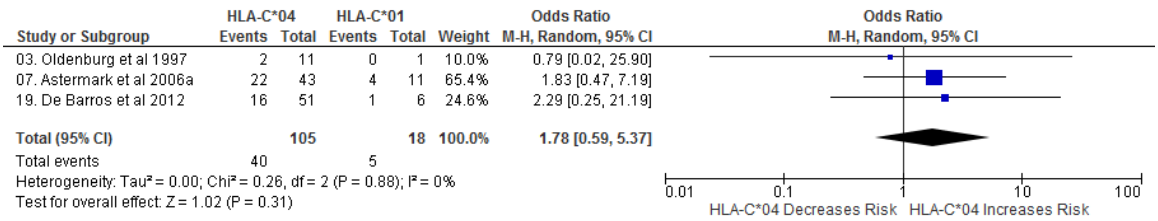
Supplementary Figure 43: Funnel plot for *HLA-C02**



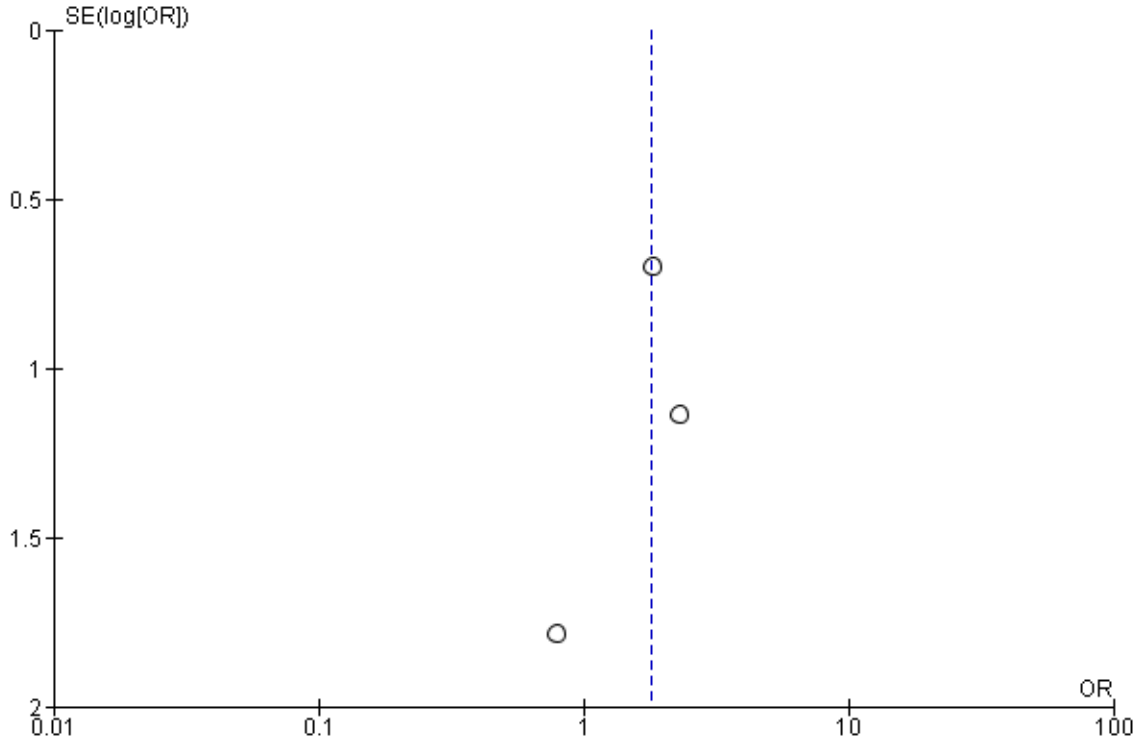
Supplementary Figure 44: Forest plot for *HLA-C03***



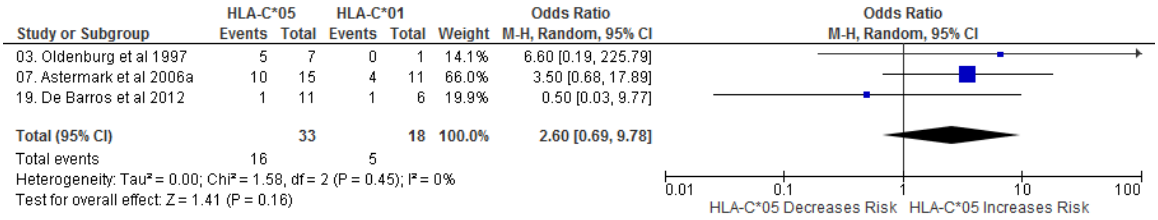
Supplementary Figure 45: Funnel plot for *HLA-C03**



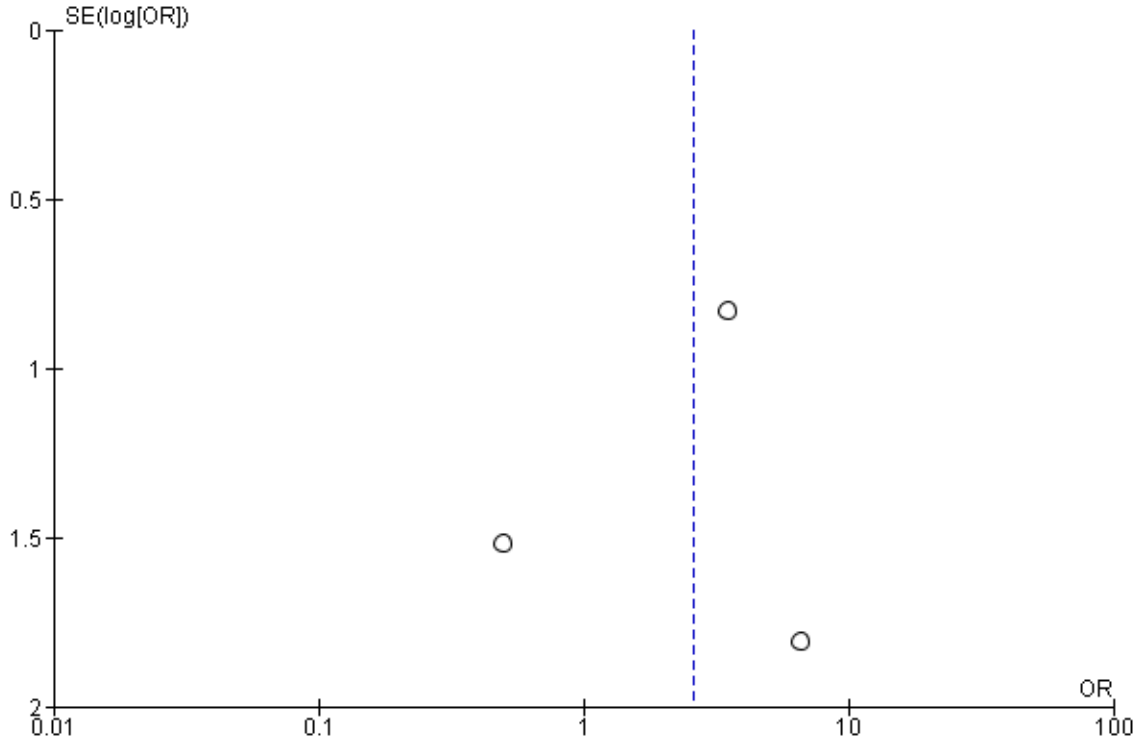
Supplementary Figure 46: Forest plot for *HLA-C04**



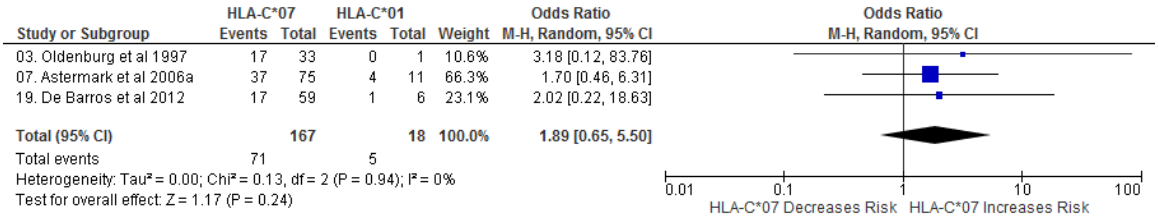
Supplementary Figure 47: Funnel plot for *HLA-C04**



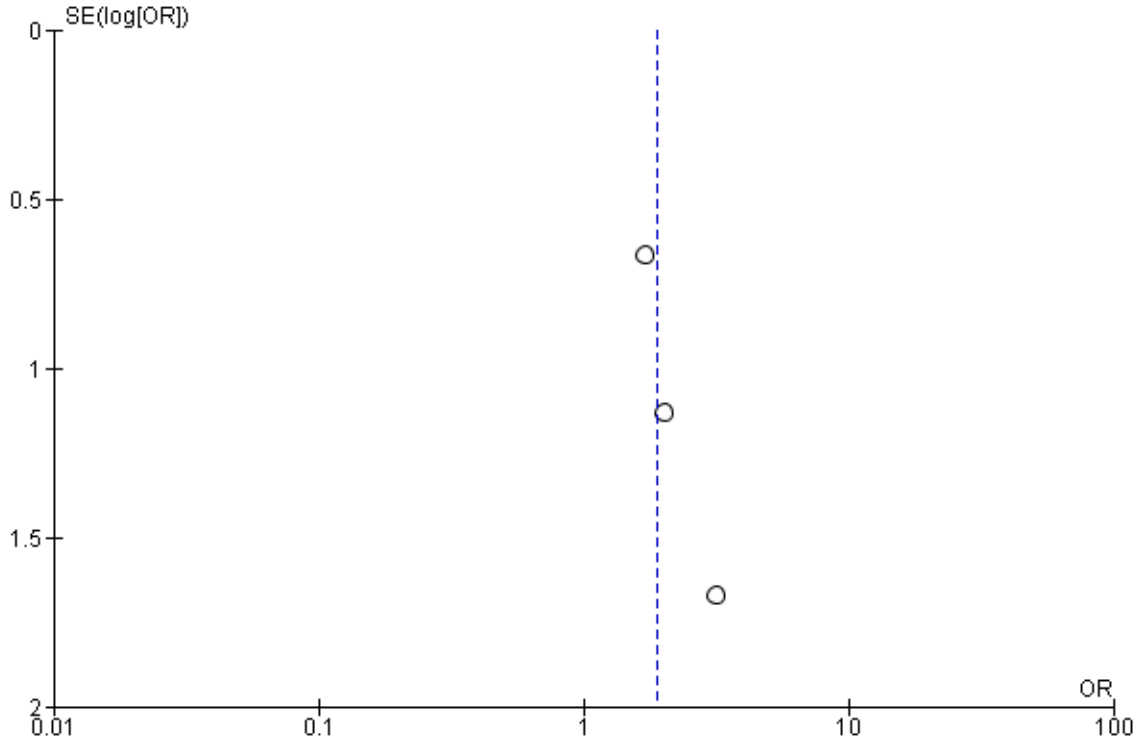
Supplementary Figure 48: Forest plot for *HLA-C05**



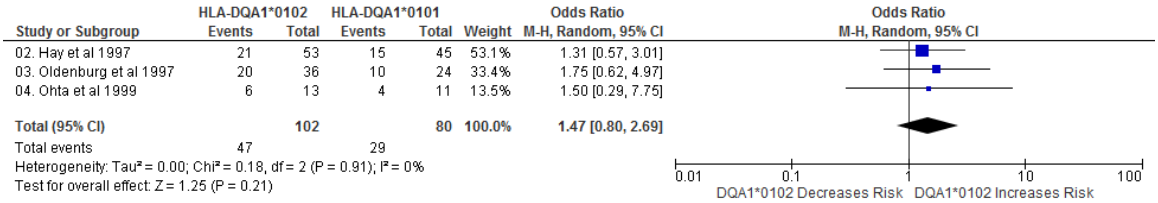
Supplementary Figure 49: Funnel plot for *HLA-C05**



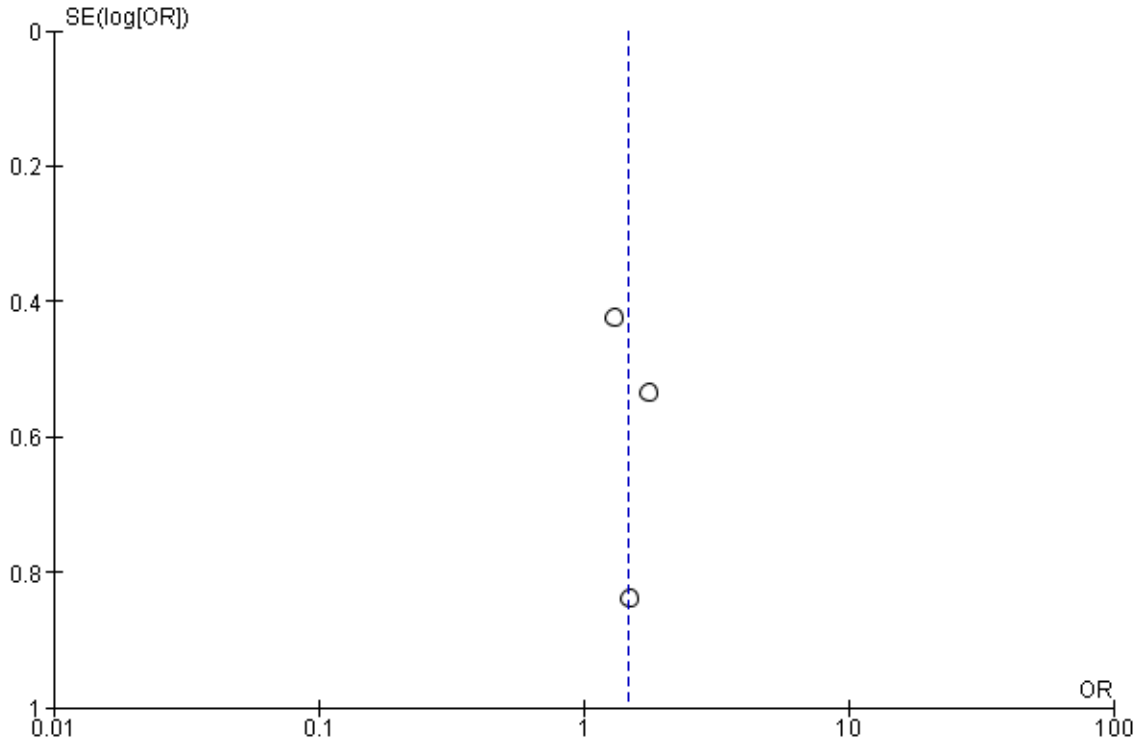
Supplementary Figure 50: Forest plot for *HLA-C07**



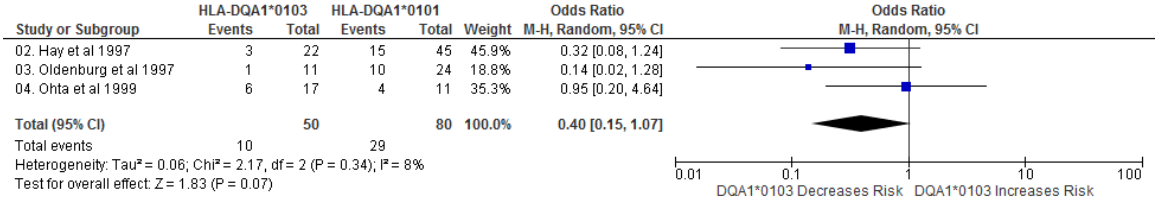
Supplementary Figure 51: Funnel plot for *HLA-C07**



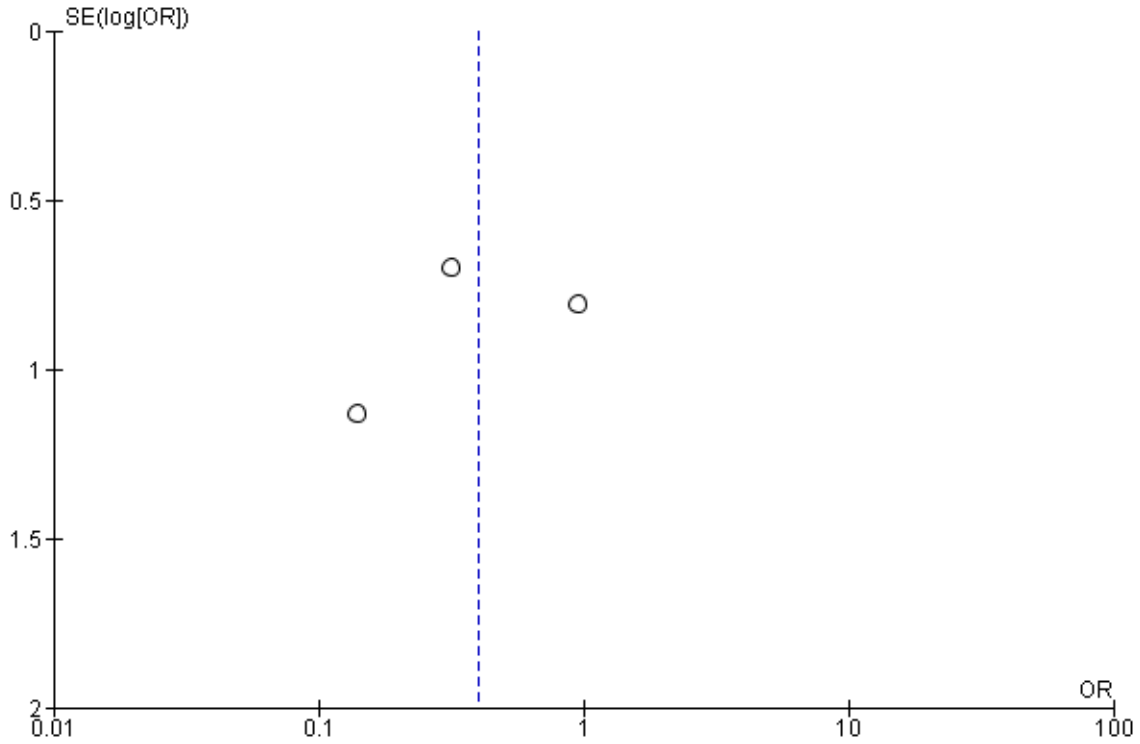
Supplementary Figure 52: Forest plot for *HLA-DQA1*01:02*



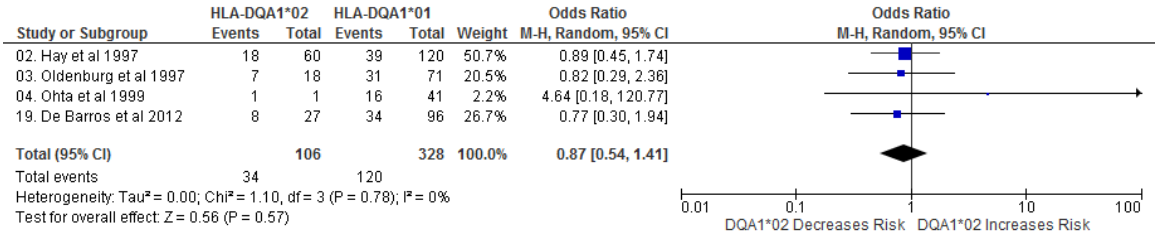
Supplementary Figure 53: Funnel plot for *HLA- DQA1*01:02*



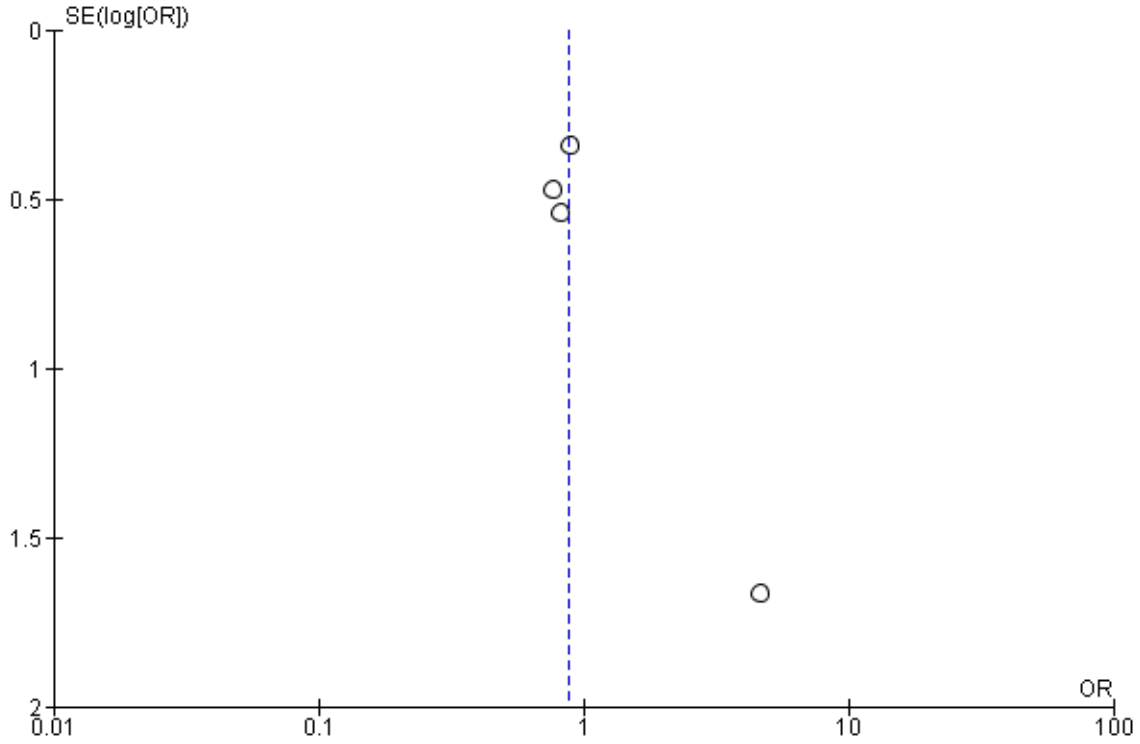
Supplementary Figure 54: Forest plot for *HLA-DQA1*01:03*



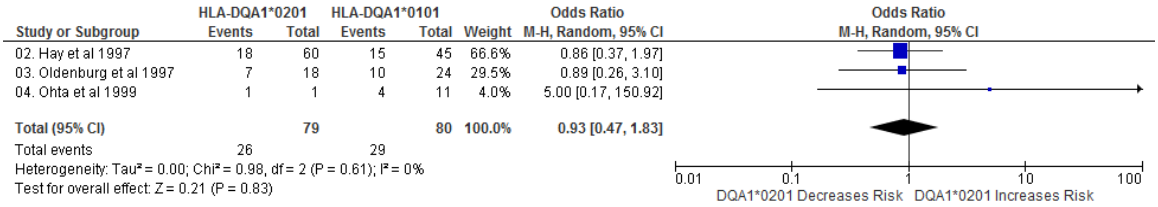
Supplementary Figure 55: Funnel plot for *HLA- DQA1*01:03*



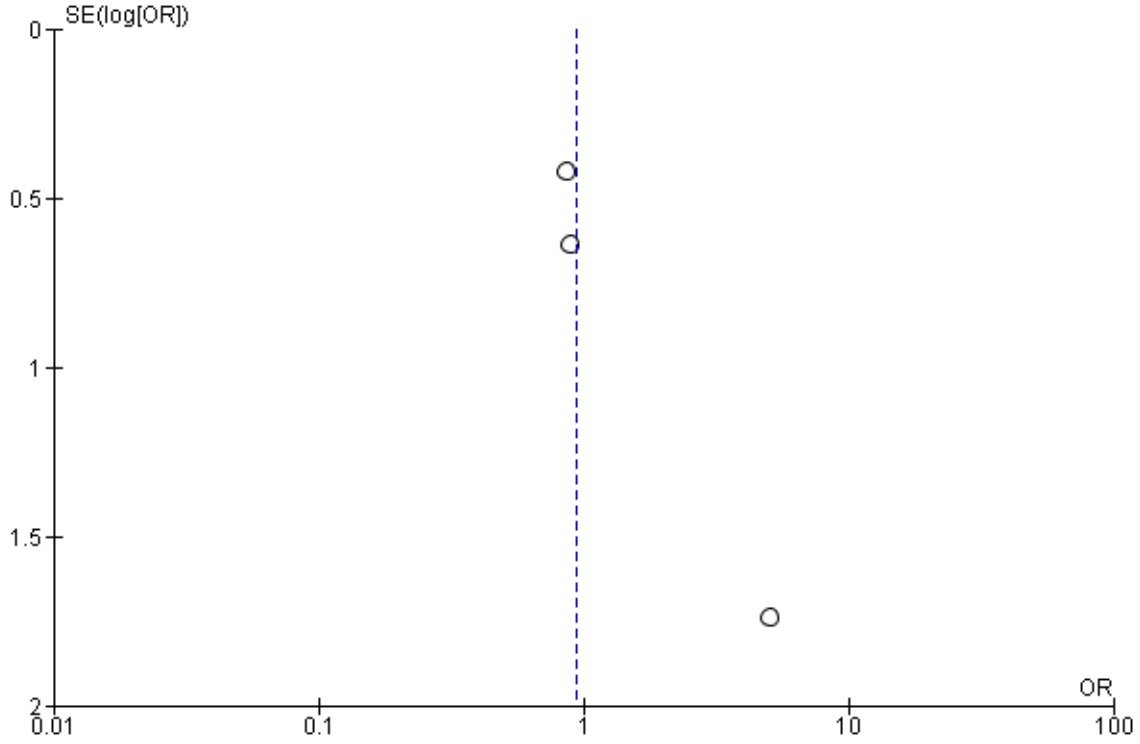
Supplementary Figure 56: Forest plot for *HLA-DQA1*02*



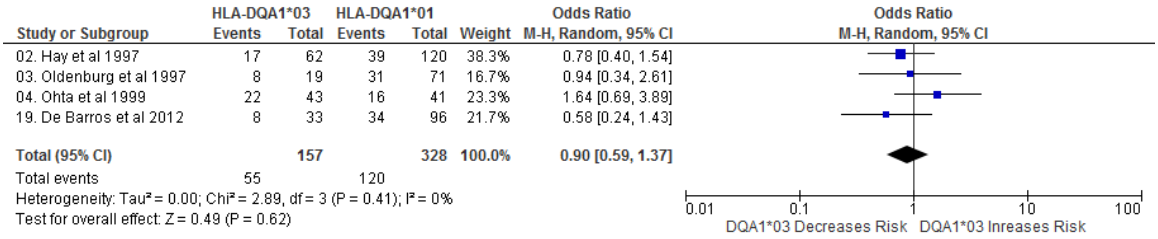
Supplementary Figure 57: Funnel plot for *HLA- DQA1*02*



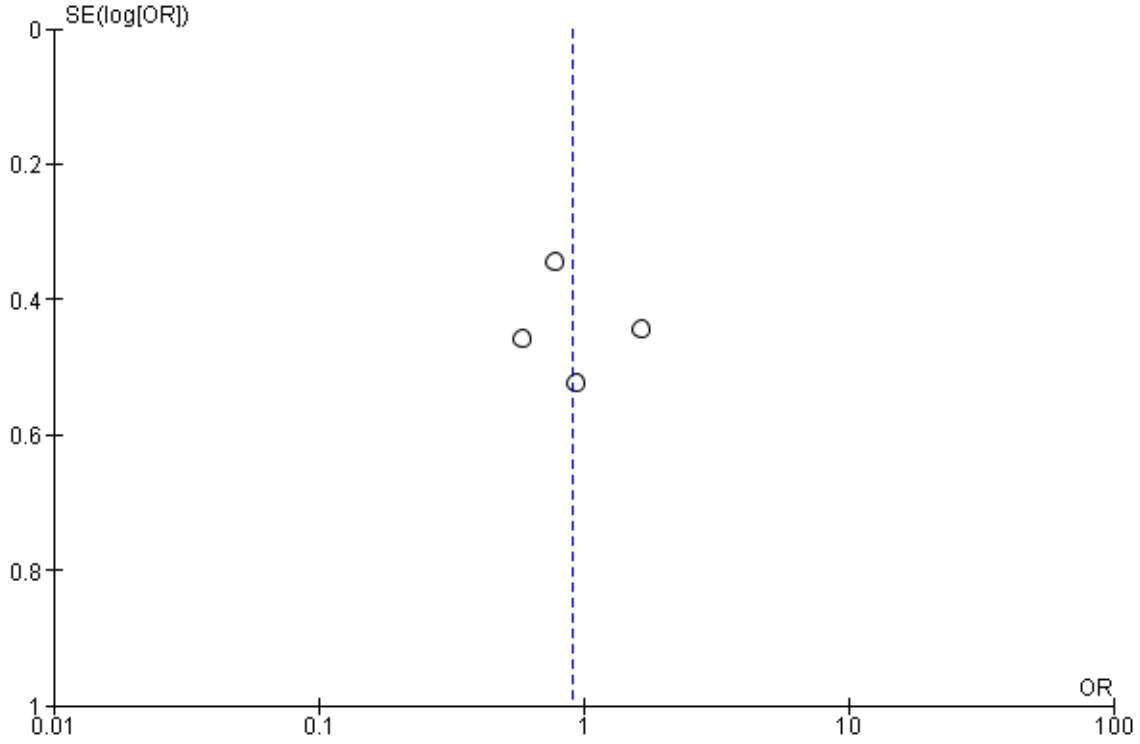
Supplementary Figure 58: Forest plot for *HLA-DQA102:01***



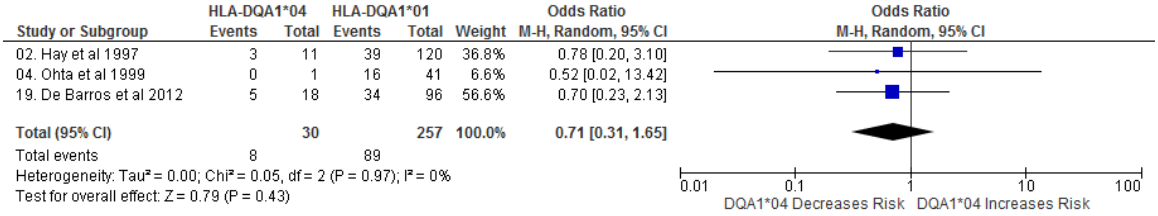
Supplementary Figure 59: Funnel plot for *HLA- DQA102:01**



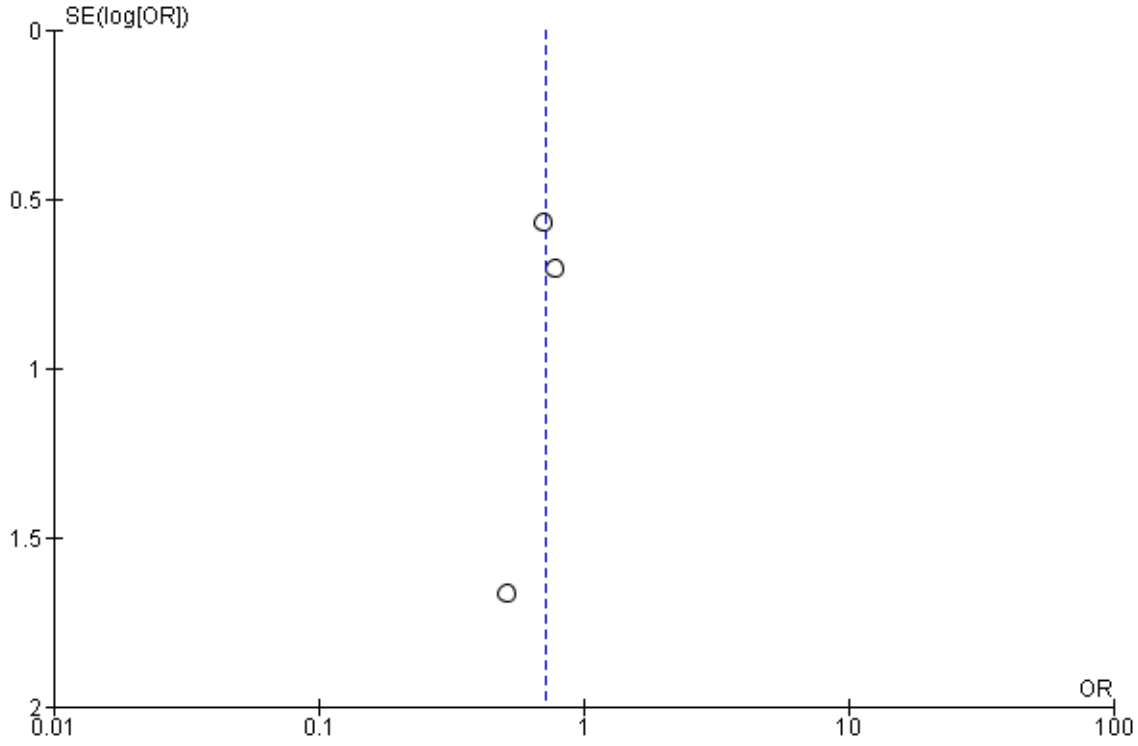
Supplementary Figure 60: Forest plot for *HLA-DQA1*03*



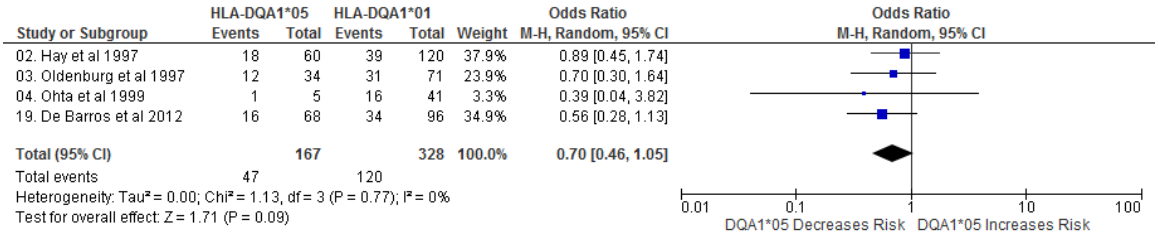
Supplementary Figure 61: Funnel plot for *HLA- DQA1*03*



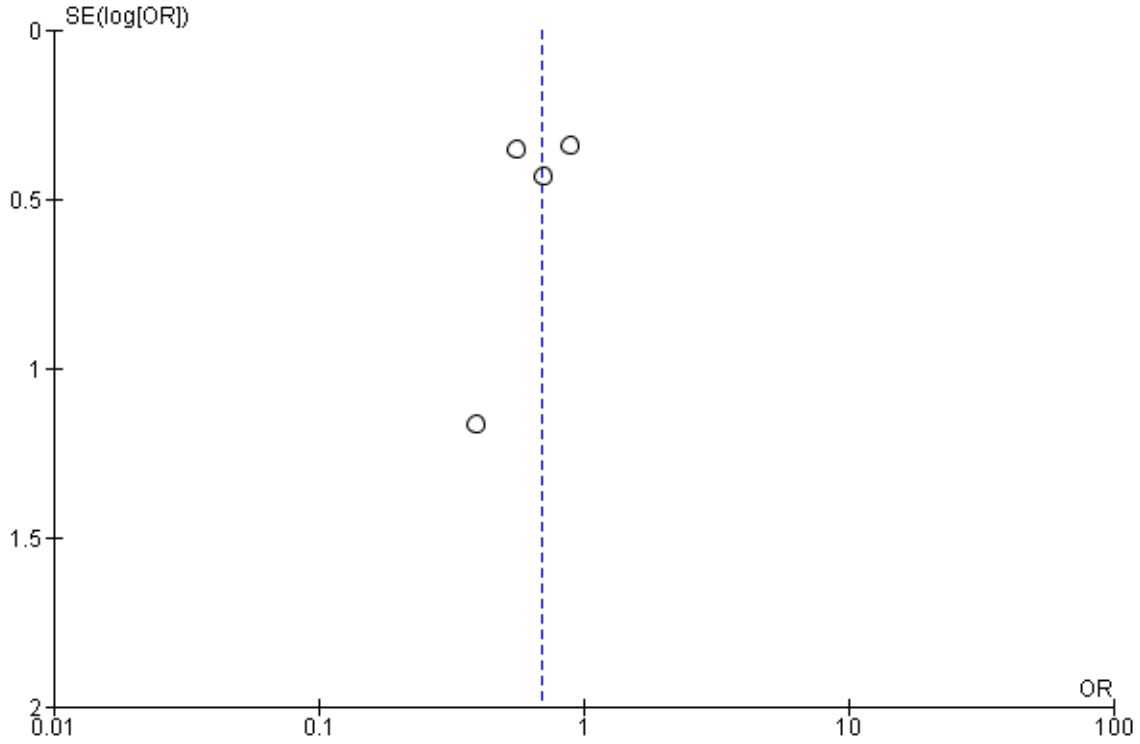
Supplementary Figure 62: Forest plot for *HLA-DQA104***



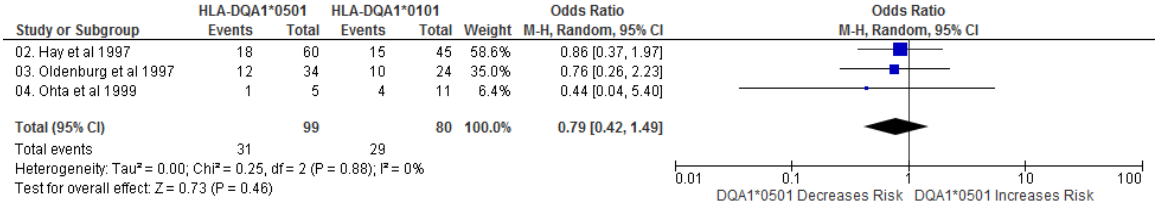
Supplementary Figure 63: Funnel plot for *HLA- DQA104**



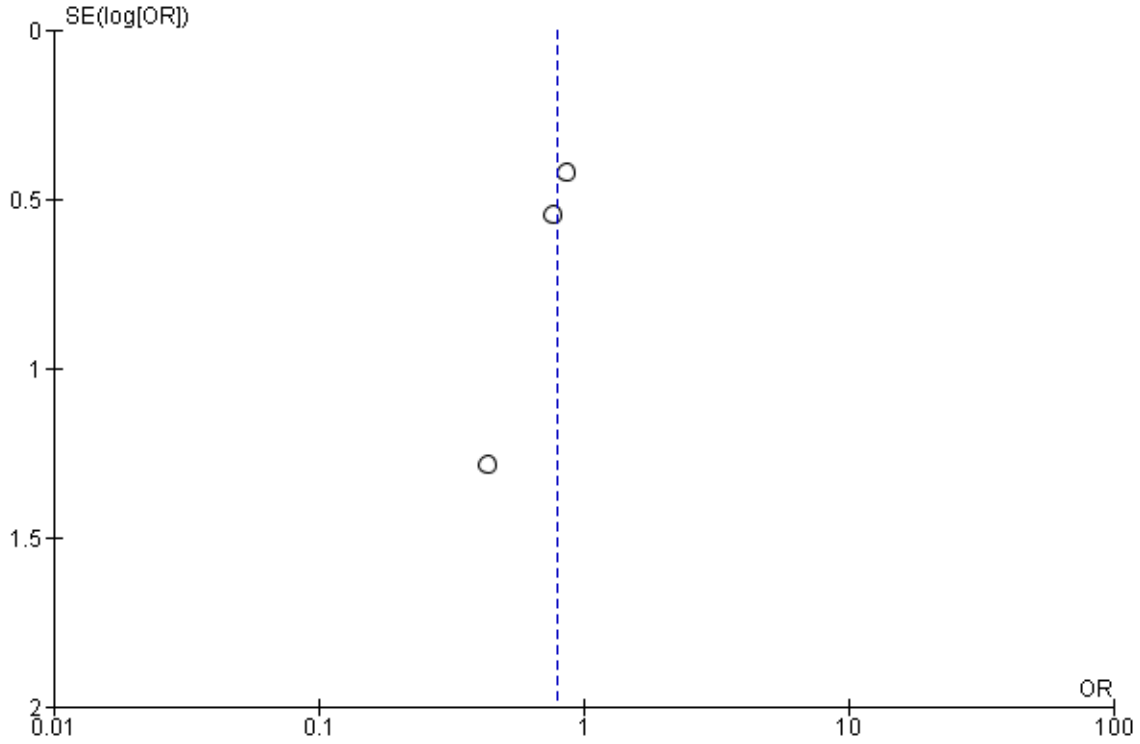
Supplementary Figure 64: Forest plot for *HLA-DQA1*05*



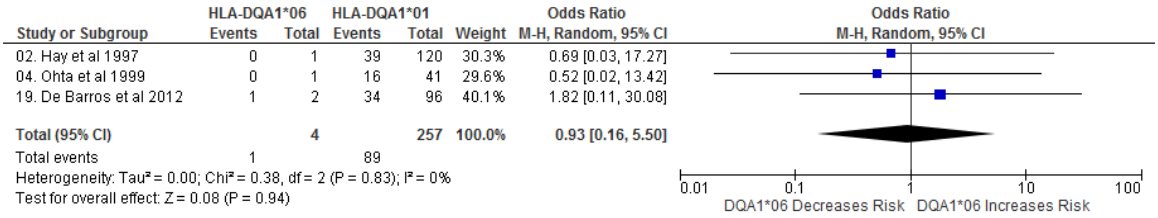
Supplementary Figure 65: Funnel plot for *HLA- DQA1*05*



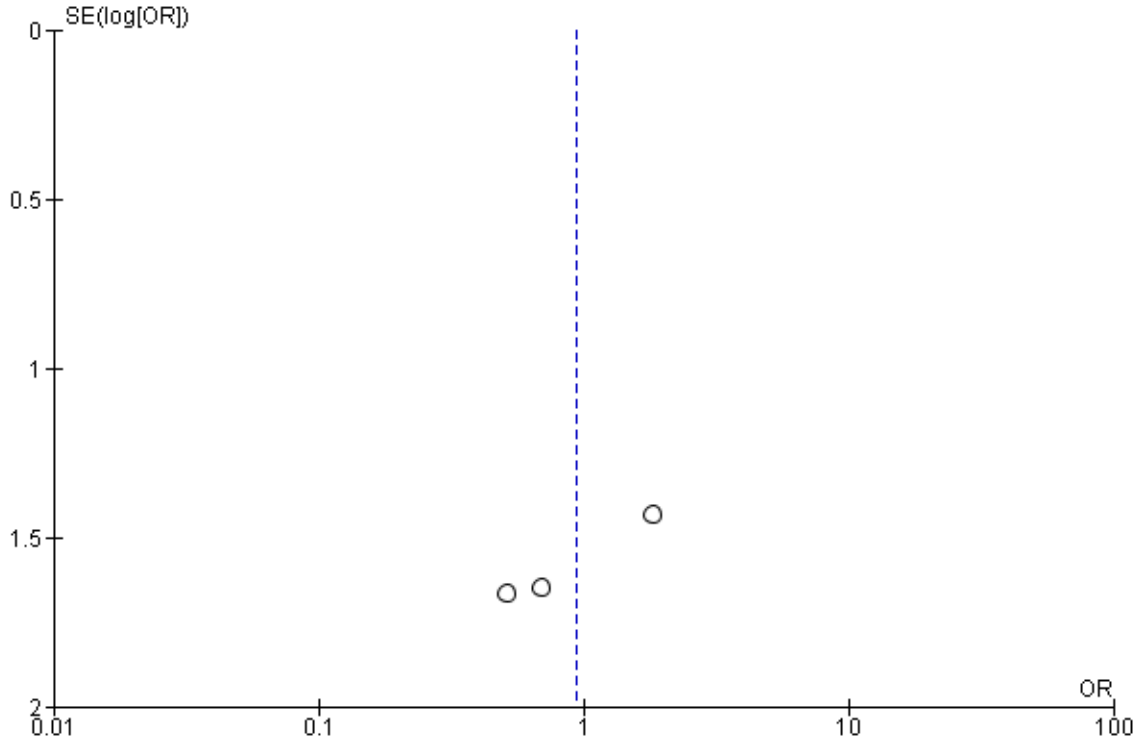
Supplementary Figure 66: Forest plot for *HLA-DQA1*05:01*



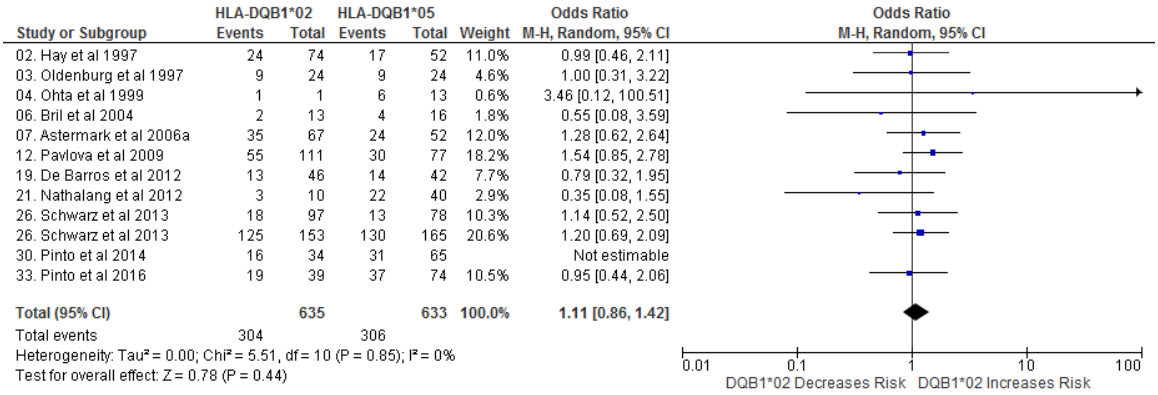
Supplementary Figure 67: Funnel plot for *HLA- DQA1*05:01*



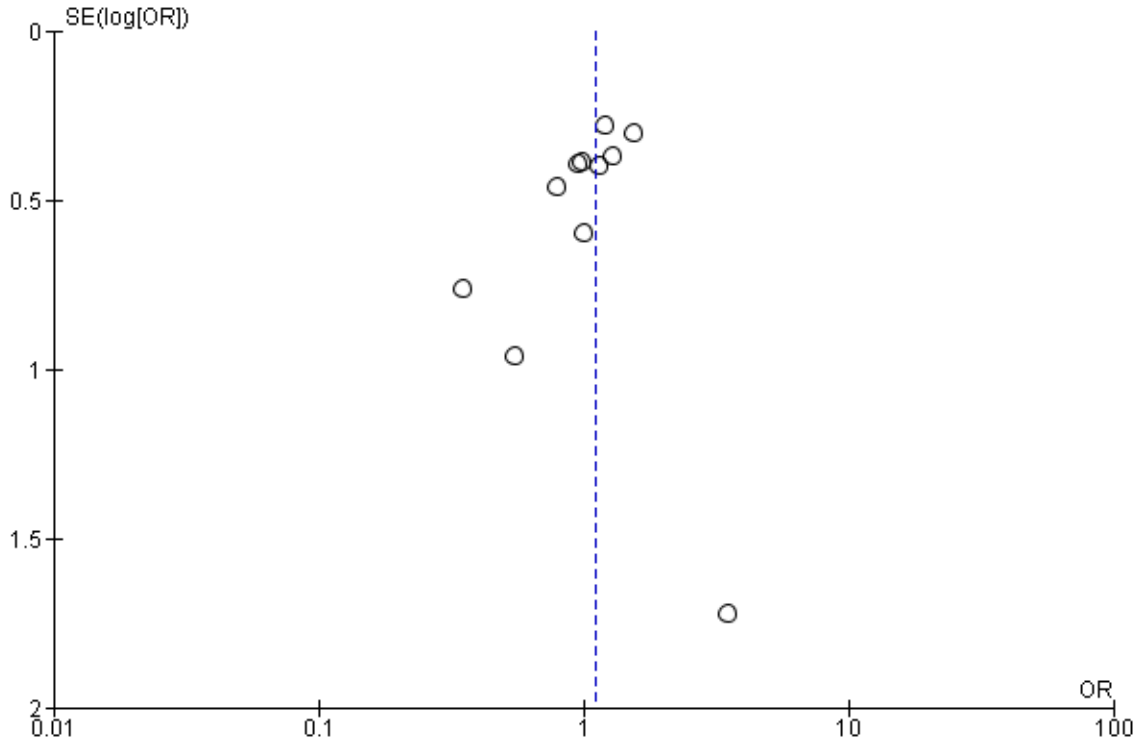
Supplementary Figure 68: Forest plot for *HLA-DQA106***



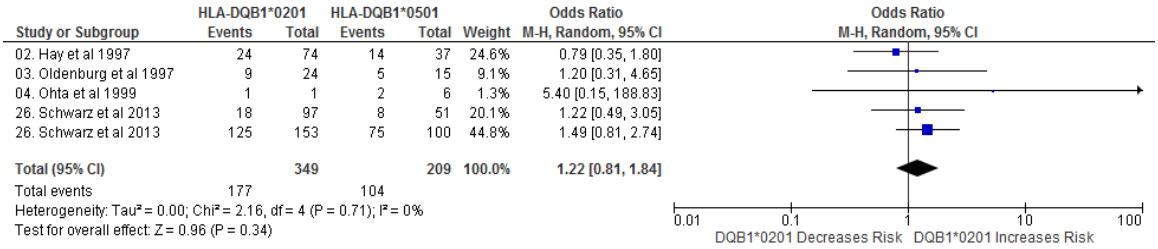
Supplementary Figure 69: Funnel plot for *HLA- DQA106**



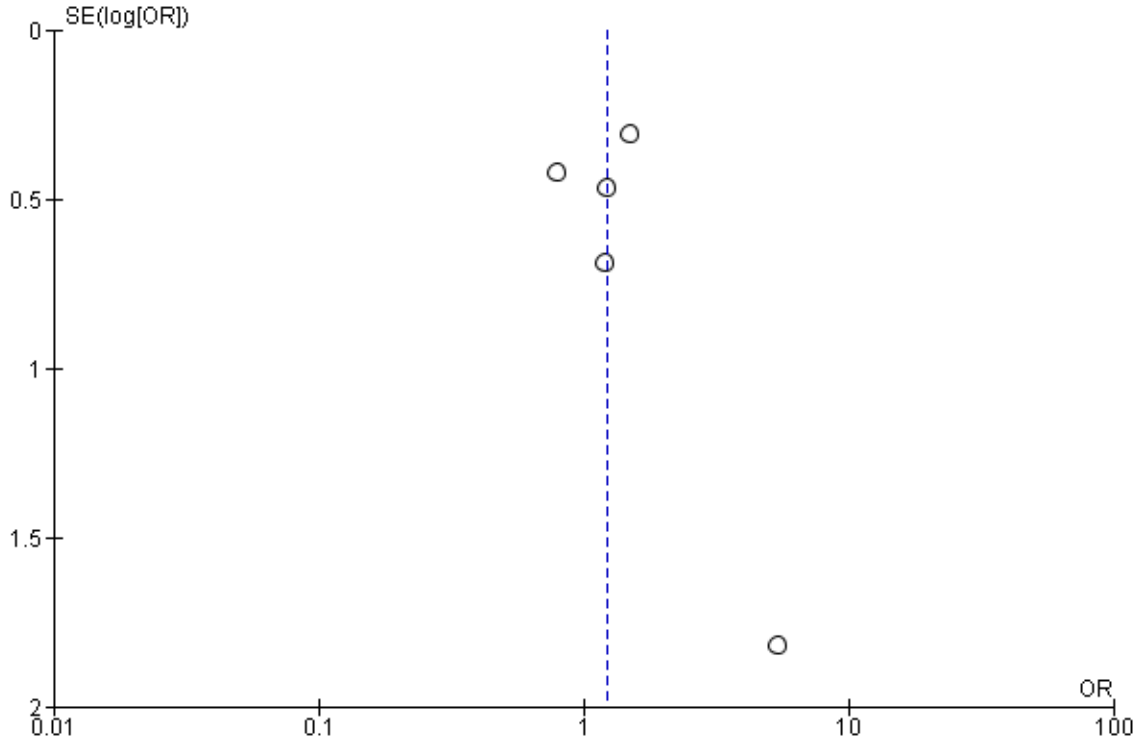
Supplementary Figure 70: Forest plot for *HLA-DQB102***



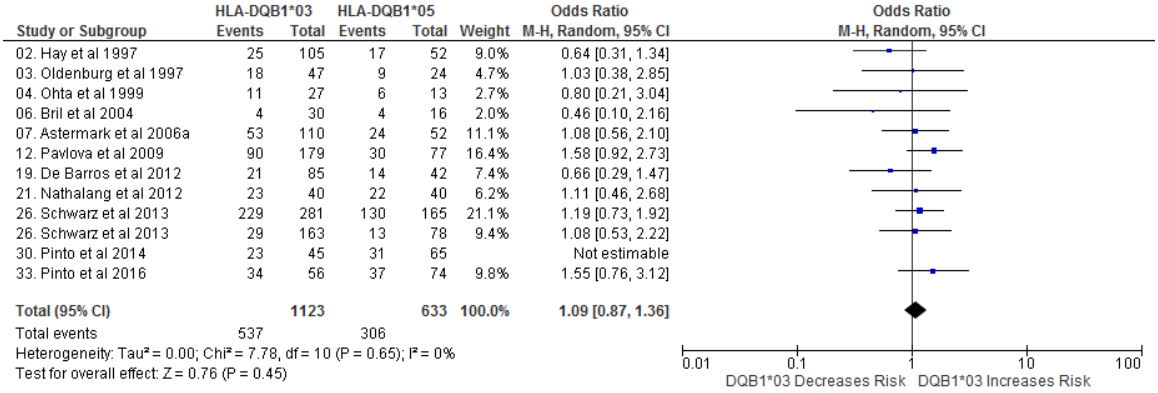
Supplementary Figure 71: Funnel plot for *HLA-DQB102***



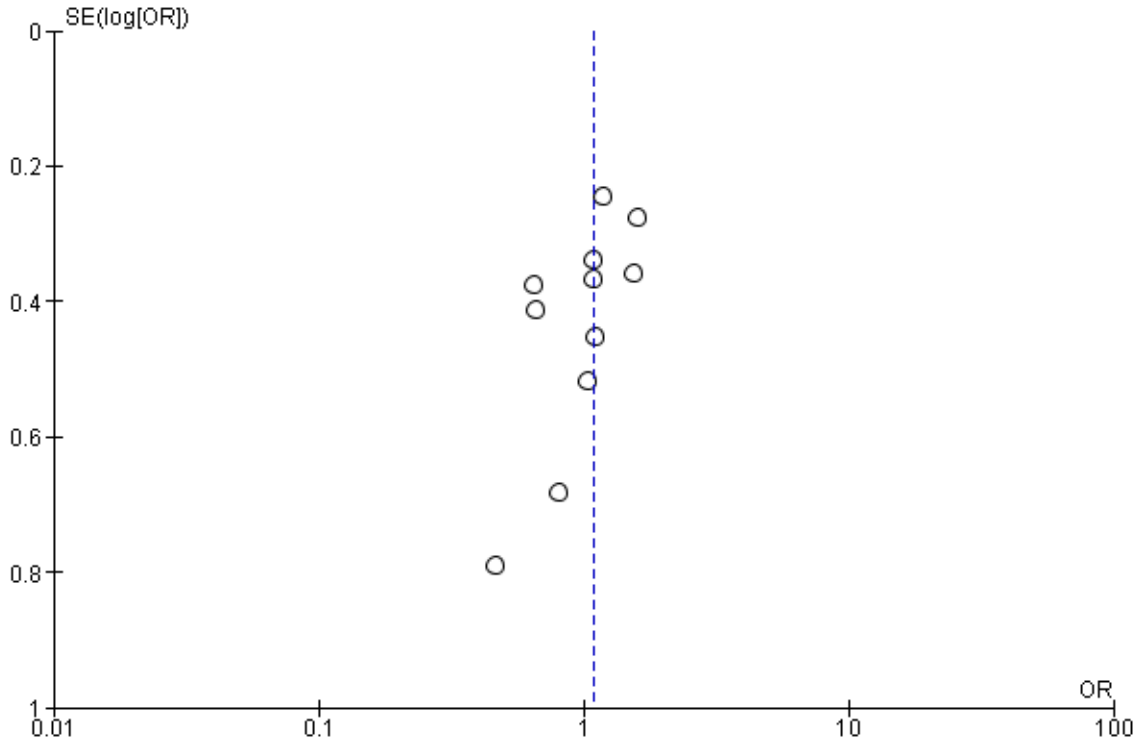
Supplementary Figure 72: Forest plot for *HLA-DQB1*02:01*



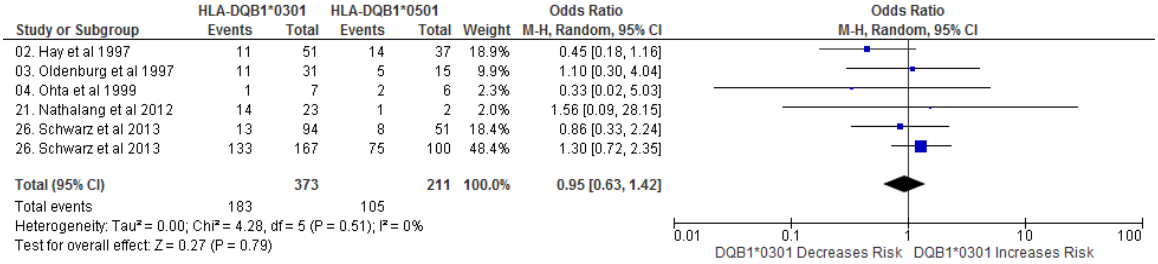
Supplementary Figure 73: Funnel plot for *HLA-DQB1*02:01*



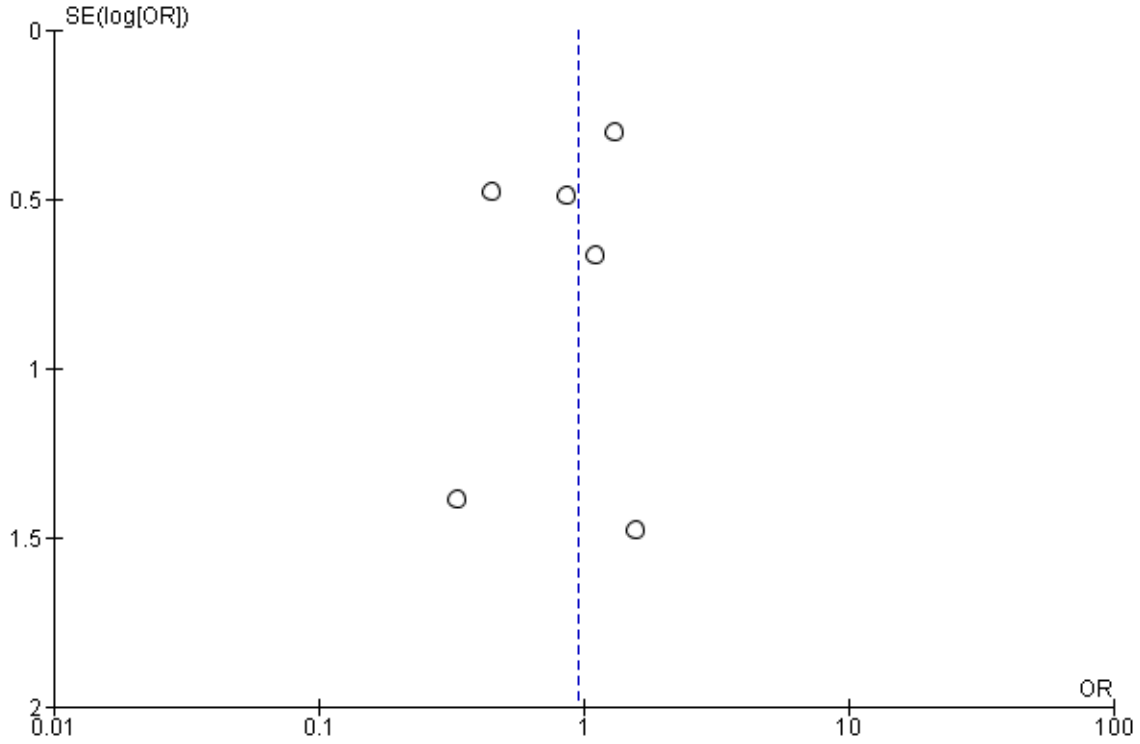
Supplementary Figure 74: Forest plot for *HLA-DQB103***



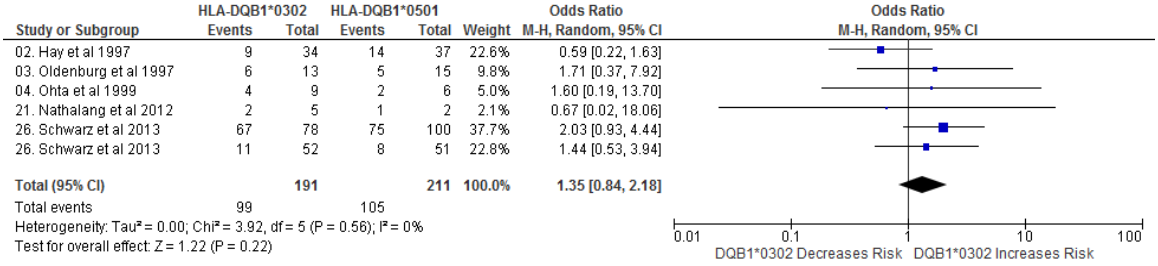
Supplementary Figure 75: Funnel plot for *HLA-DQB103**



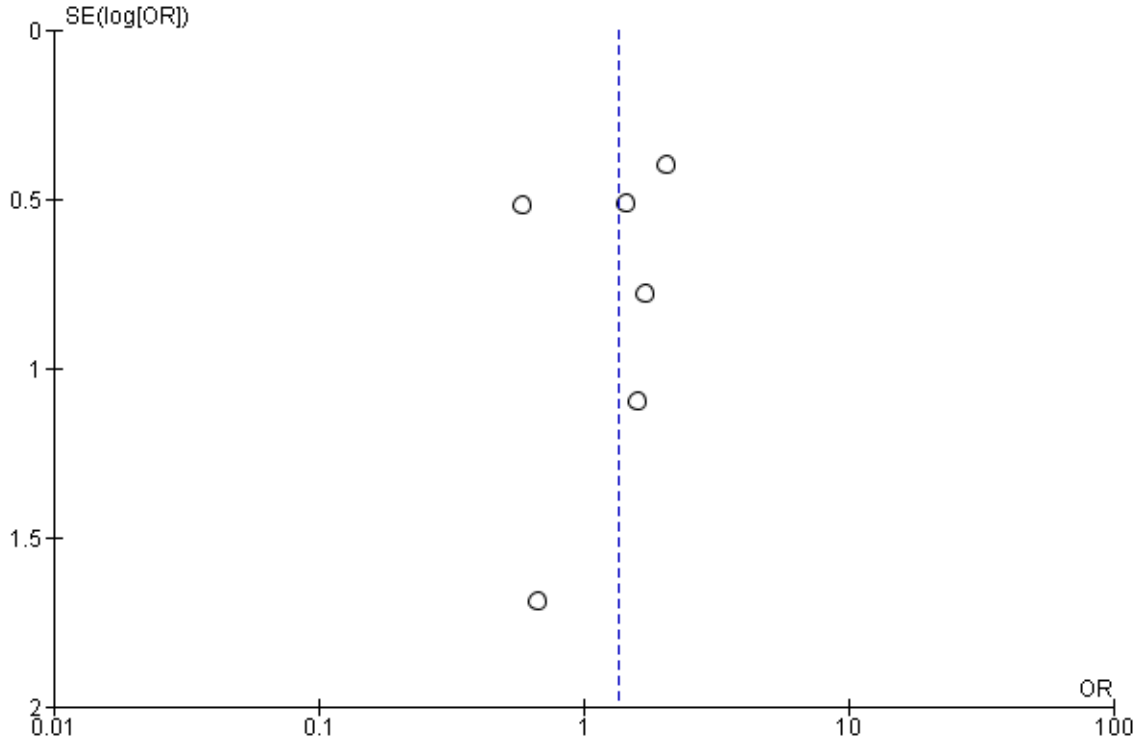
Supplementary Figure 76: Forest plot for *HLA-DQB103:01**



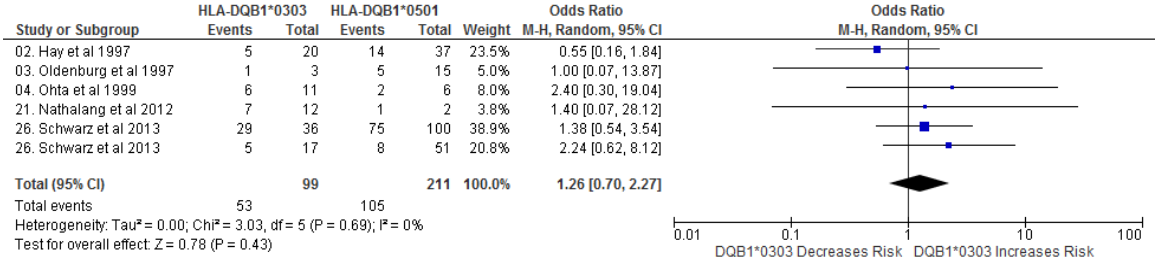
Supplementary Figure 77: Funnel plot for *HLA-DQB103:01**



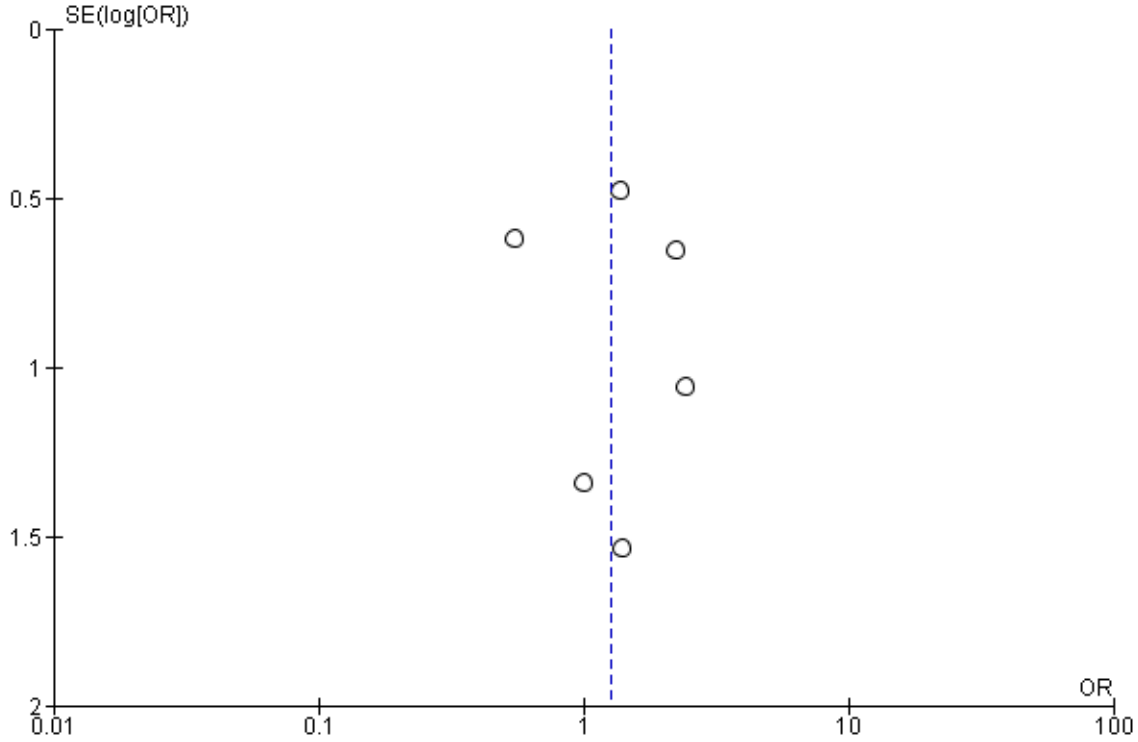
Supplementary Figure 78: Forest plot for *HLA-DQB103:02***



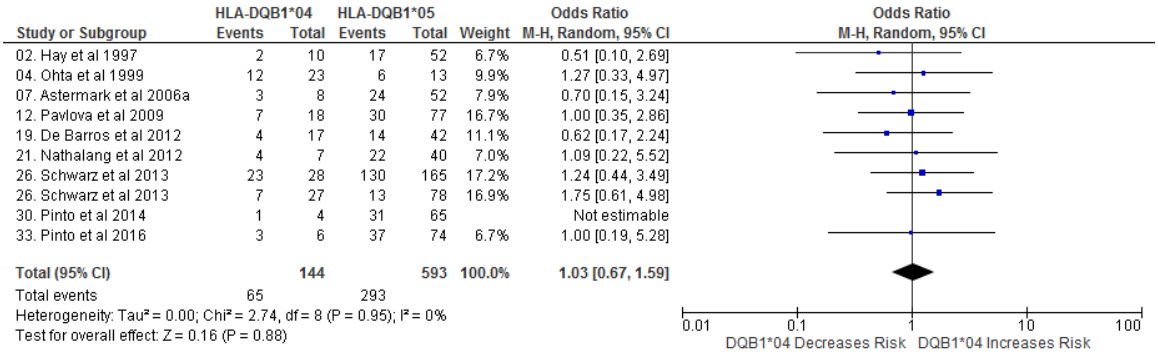
Supplementary Figure 79: Funnel plot for *HLA-DQB103:02***



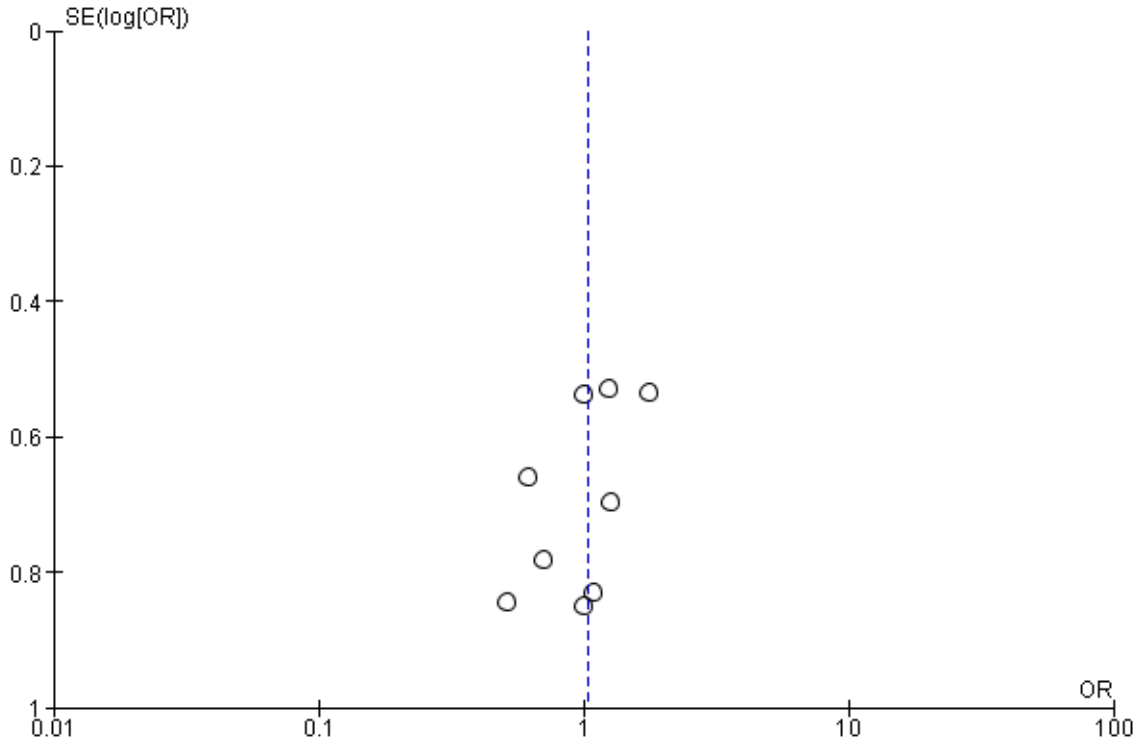
Supplementary Figure 80: Forest plot for *HLA-DQB103:03***



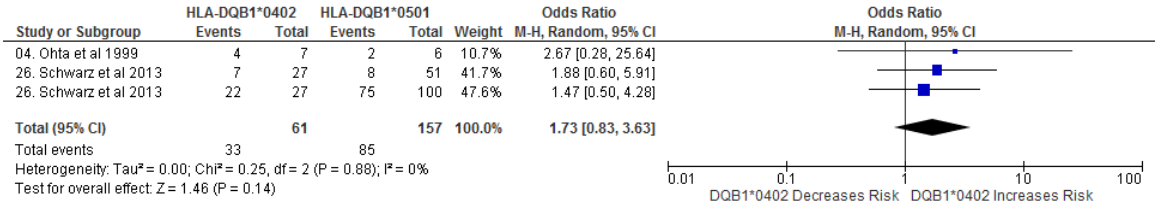
Supplementary Figure 81: Funnel plot for *HLA-DQB103:03***



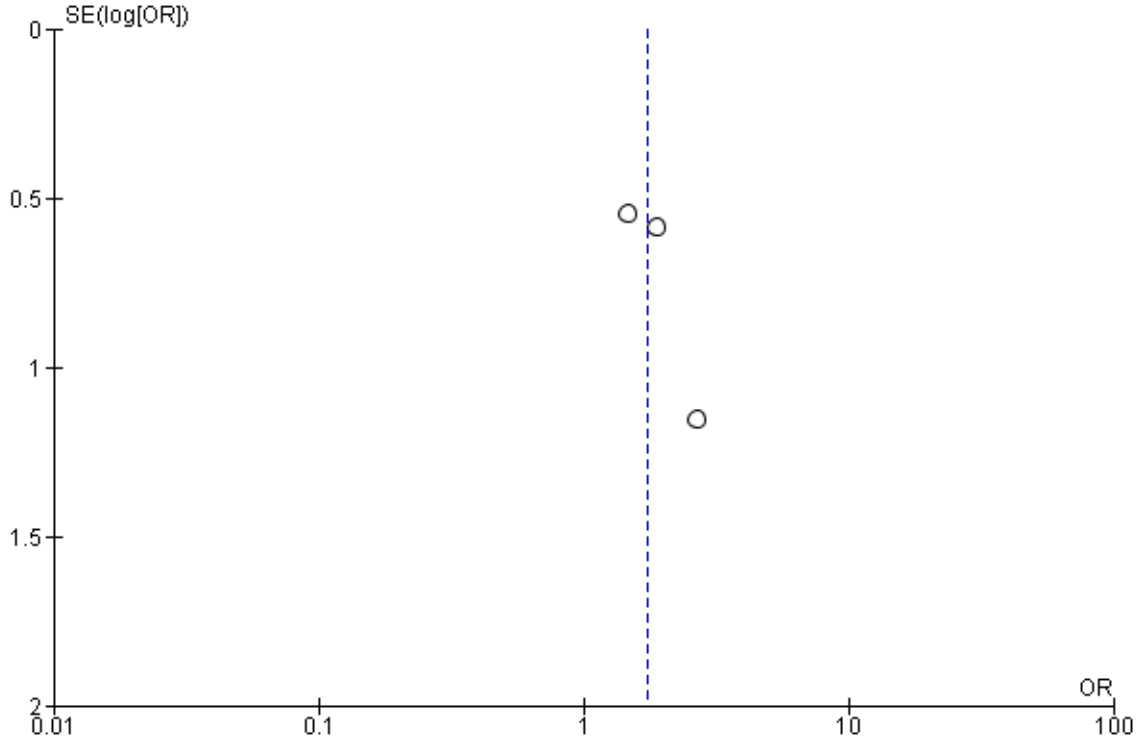
Supplementary Figure 82: Forest plot for *HLA-DQB104***



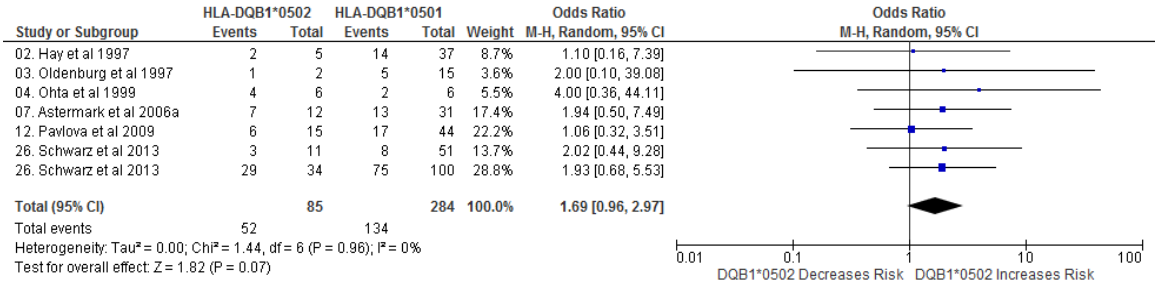
Supplementary Figure 83: Funnel plot for *HLA-DQB104**



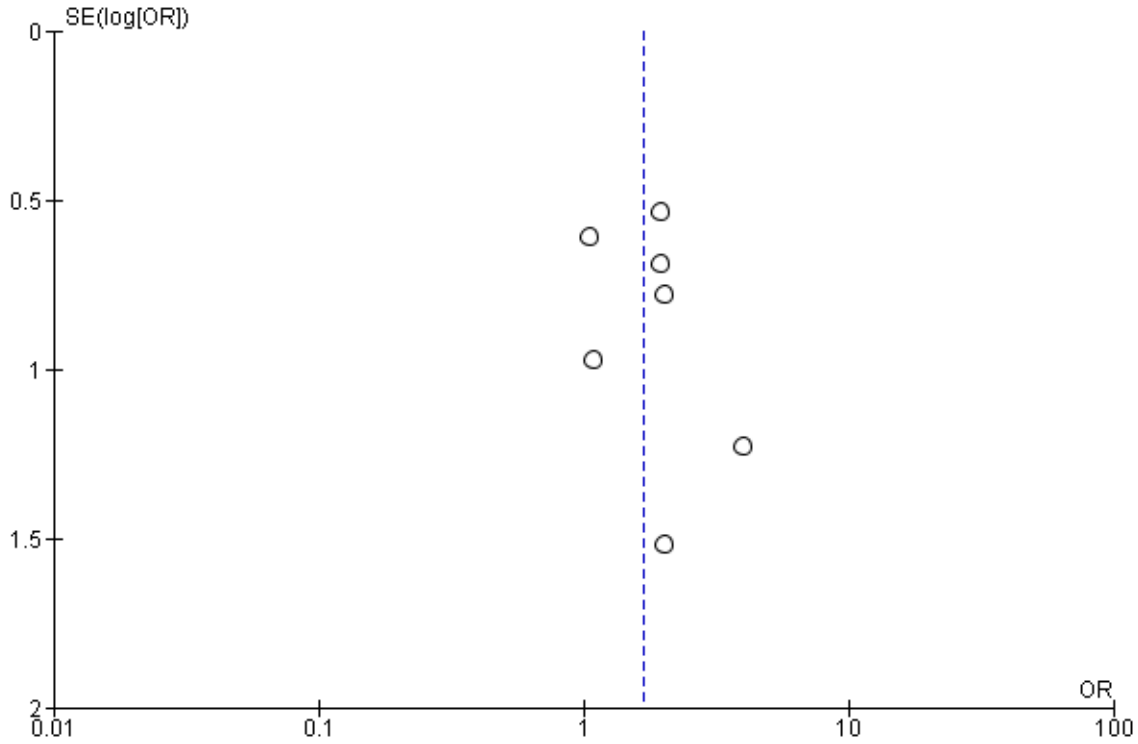
Supplementary Figure 84: Forest plot for *HLA-DQB1*04:02*



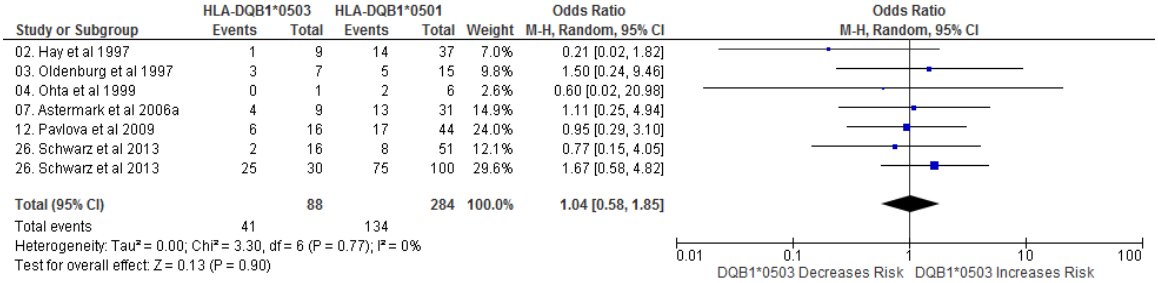
Supplementary Figure 85: Funnel plot for *HLA-DQB1*04:02*



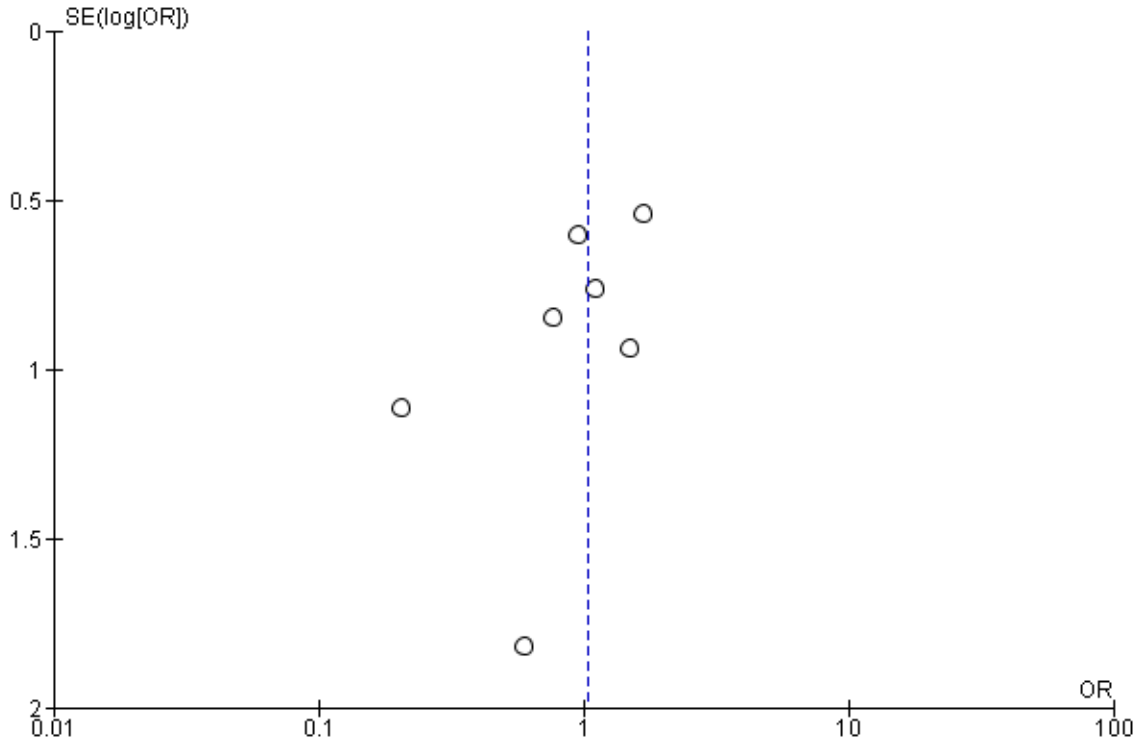
Supplementary Figure 86: Forest plot for *HLA-DQB105:02***



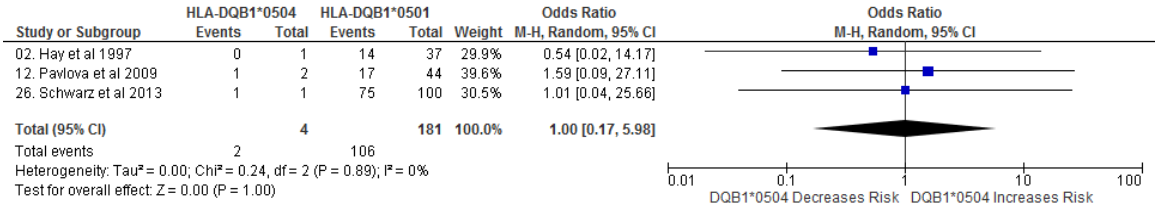
Supplementary Figure 87: Funnel plot for *HLA-DQB105:02***



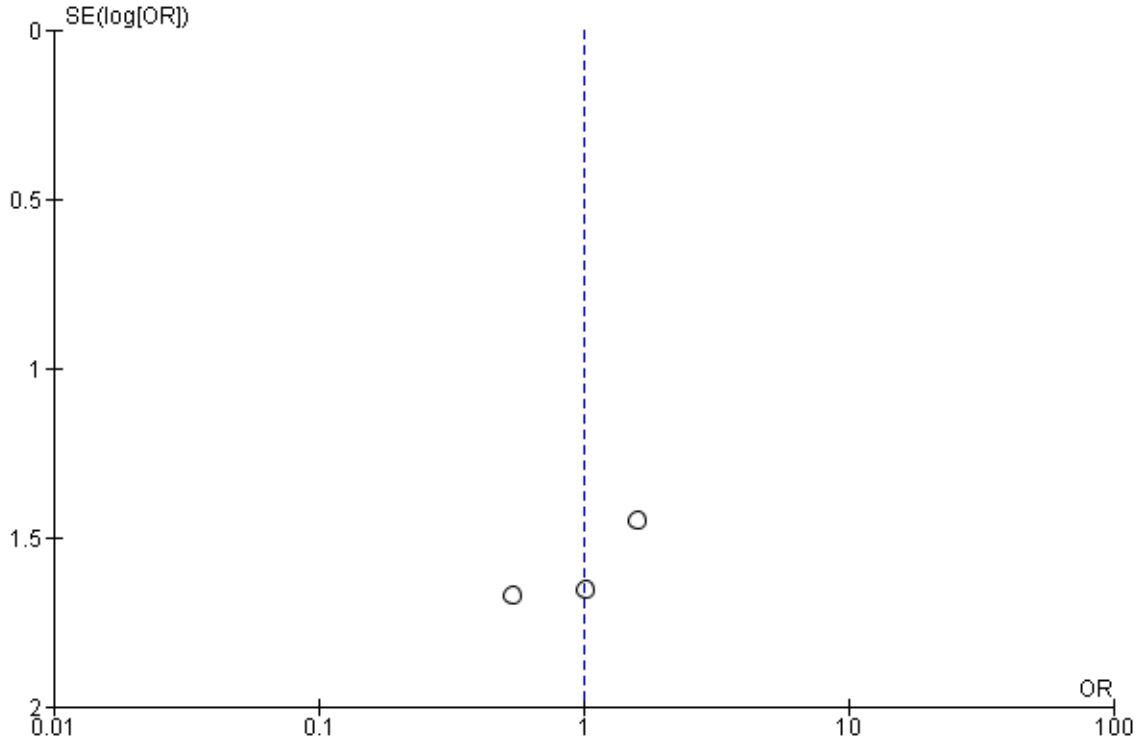
Supplementary Figure 88: Forest plot for *HLA-DQB105:03***



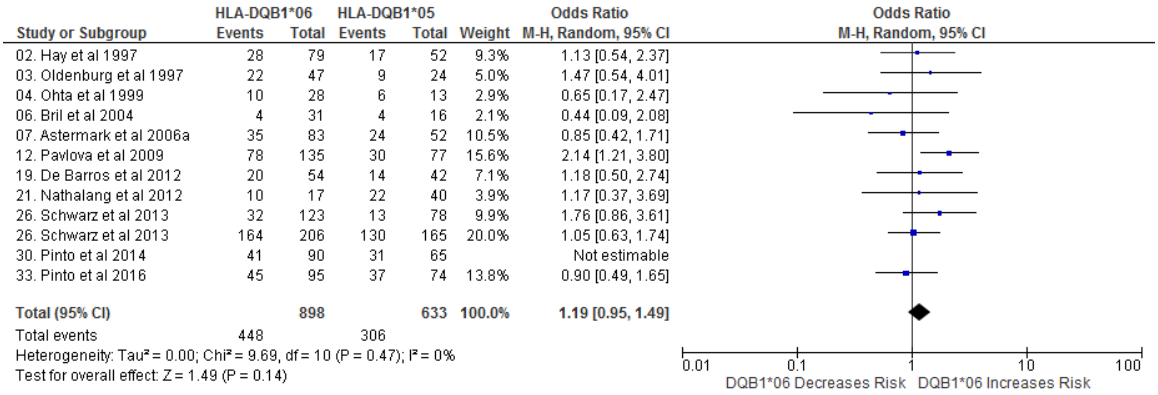
Supplementary Figure 89: Funnel plot for *HLA-DQB105:03***



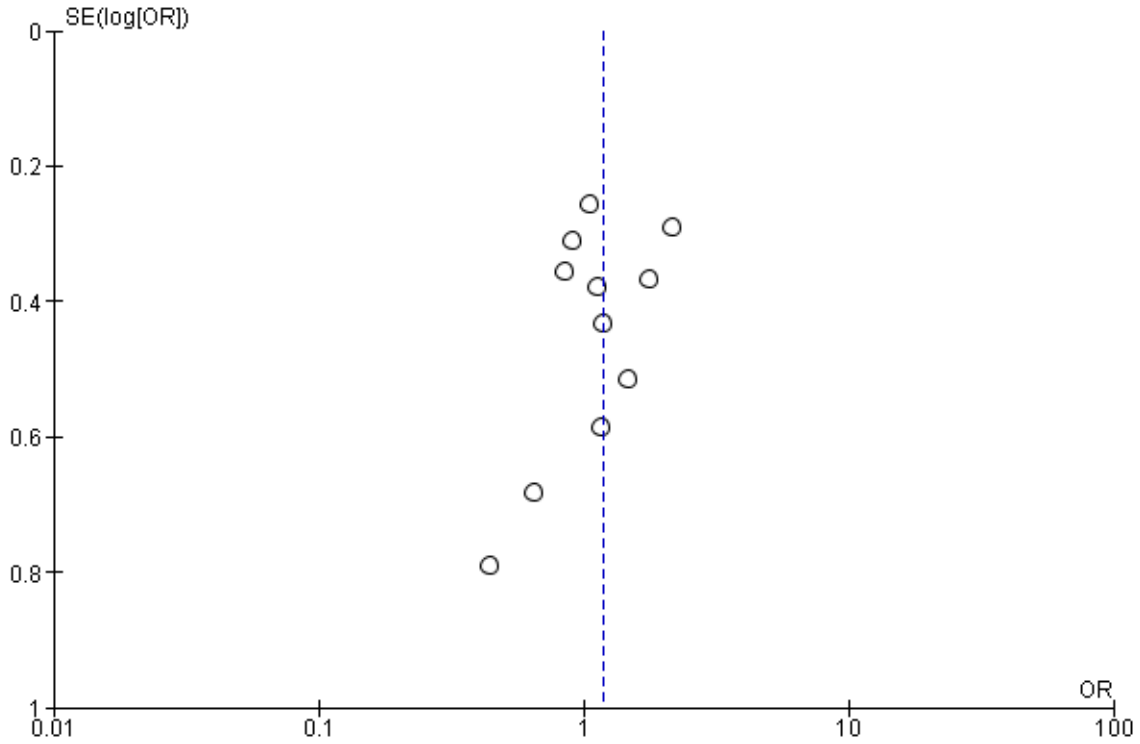
Supplementary Figure 90: Forest plot for *HLA-DQB1*05:04*



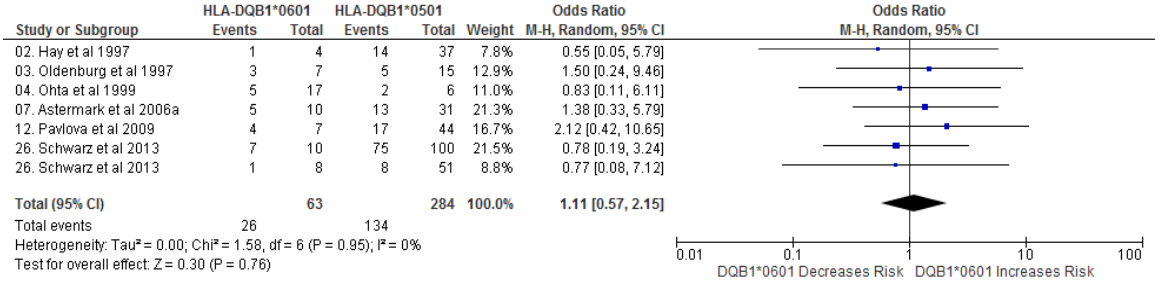
Supplementary Figure 91: Funnel plot for *HLA-DQB1*05:04*



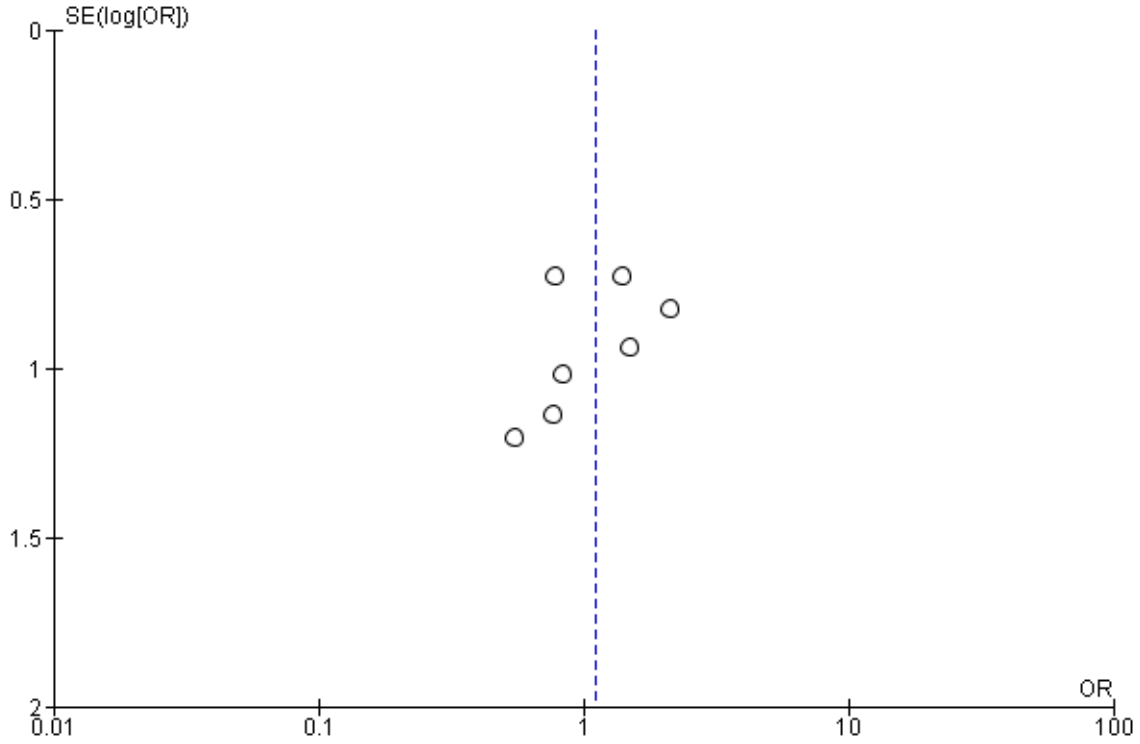
Supplementary Figure 92: Forest plot for *HLA-DQB106***



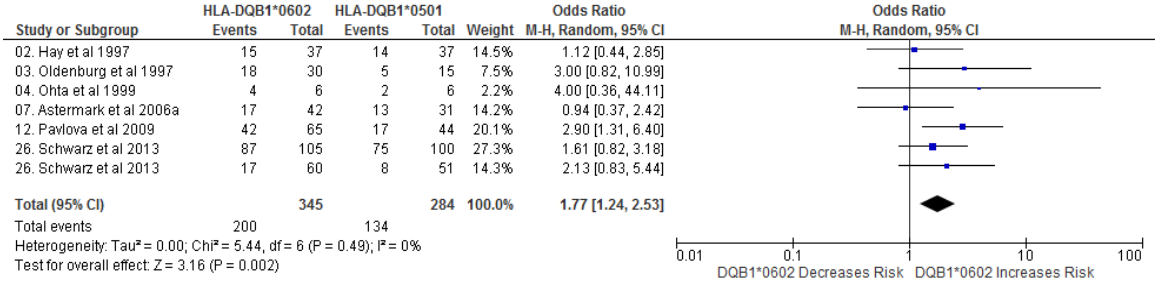
Supplementary Figure 93: Funnel plot for *HLA-DQB106**



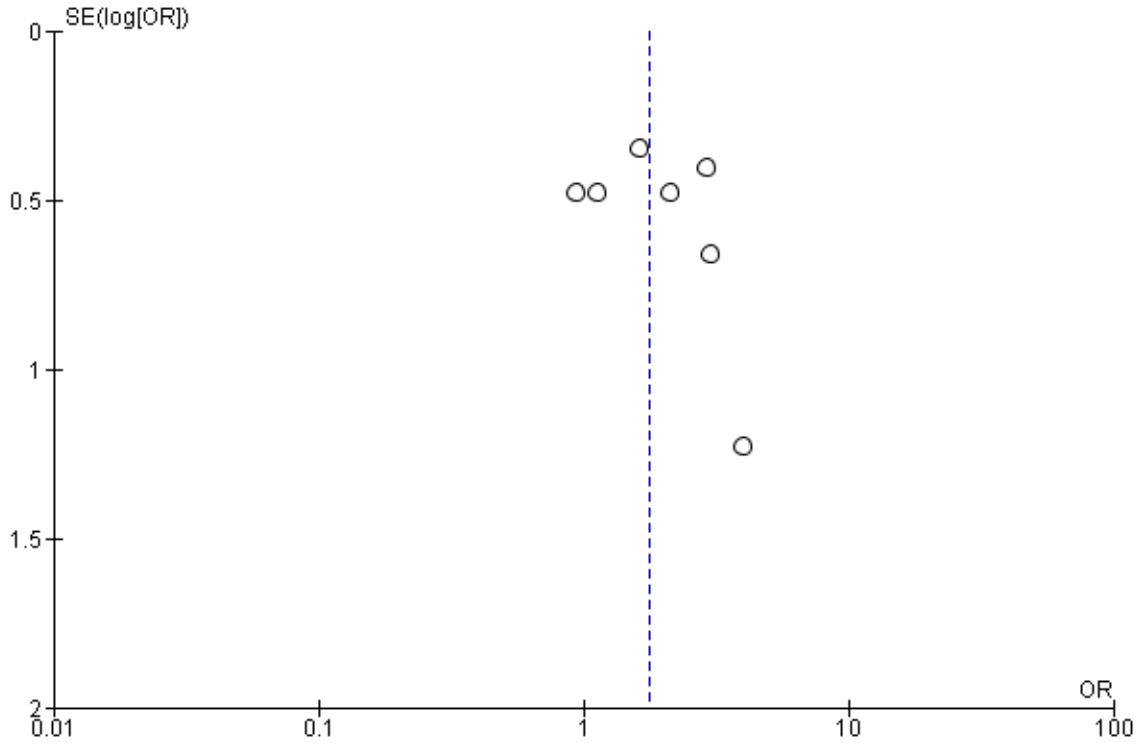
Supplementary Figure 94: Forest plot for *HLA-DQB106:01***



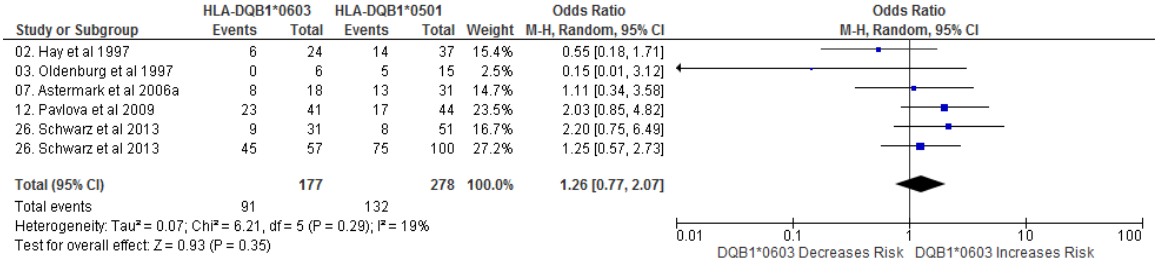
Supplementary Figure 95: Funnel plot for *HLA-DQB106:01***



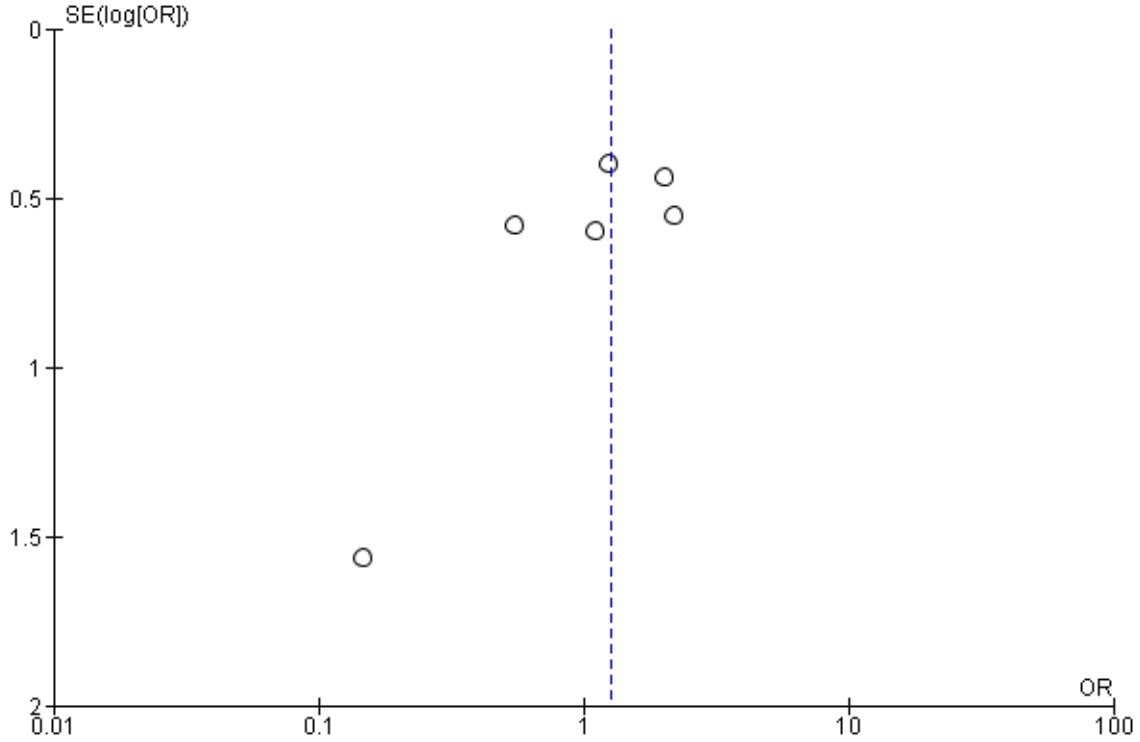
Supplementary Figure 96: Forest plot for *HLA-DQB106:02***



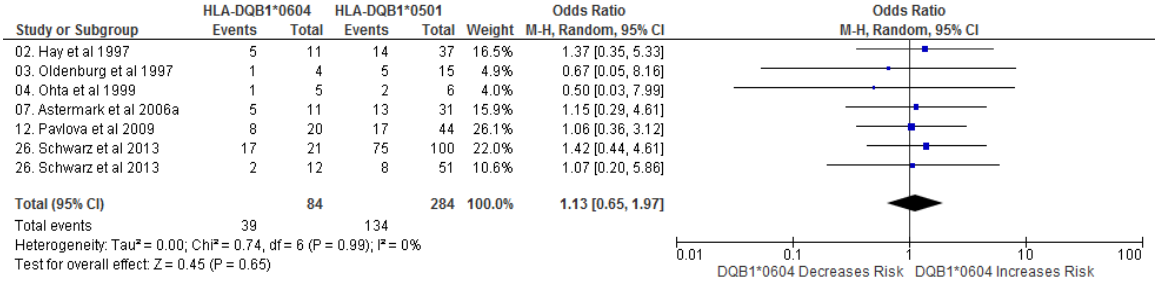
Supplementary Figure 97: Funnel plot for *HLA-DQB106:02***



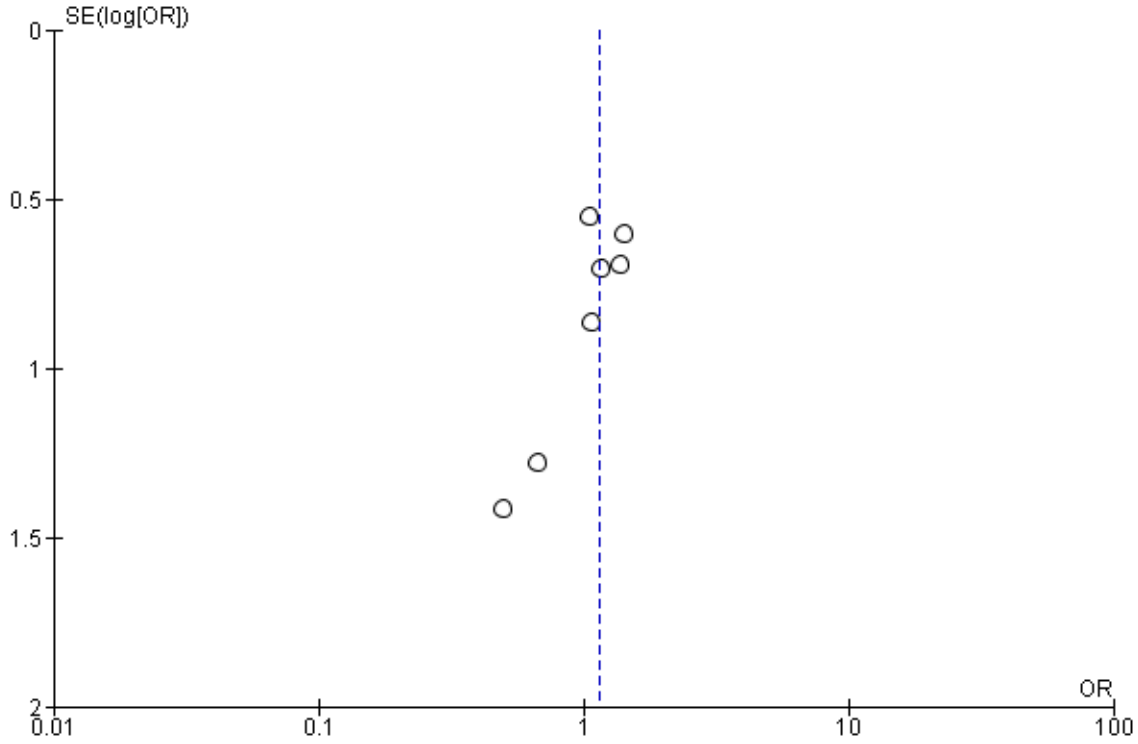
Supplementary Figure 98: Forest plot for *HLA-DQB1*06:03*



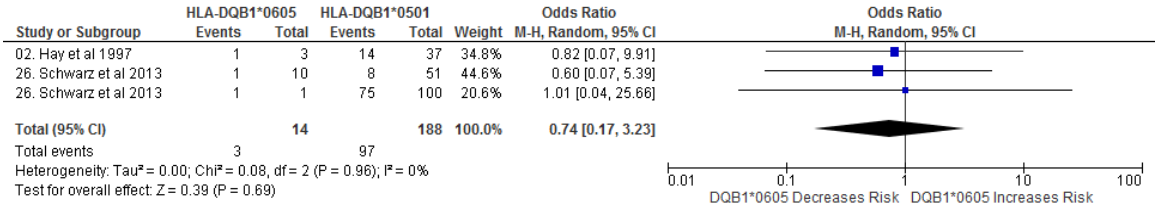
Supplementary Figure 99: Funnel plot for *HLA-DQB1*06:03*



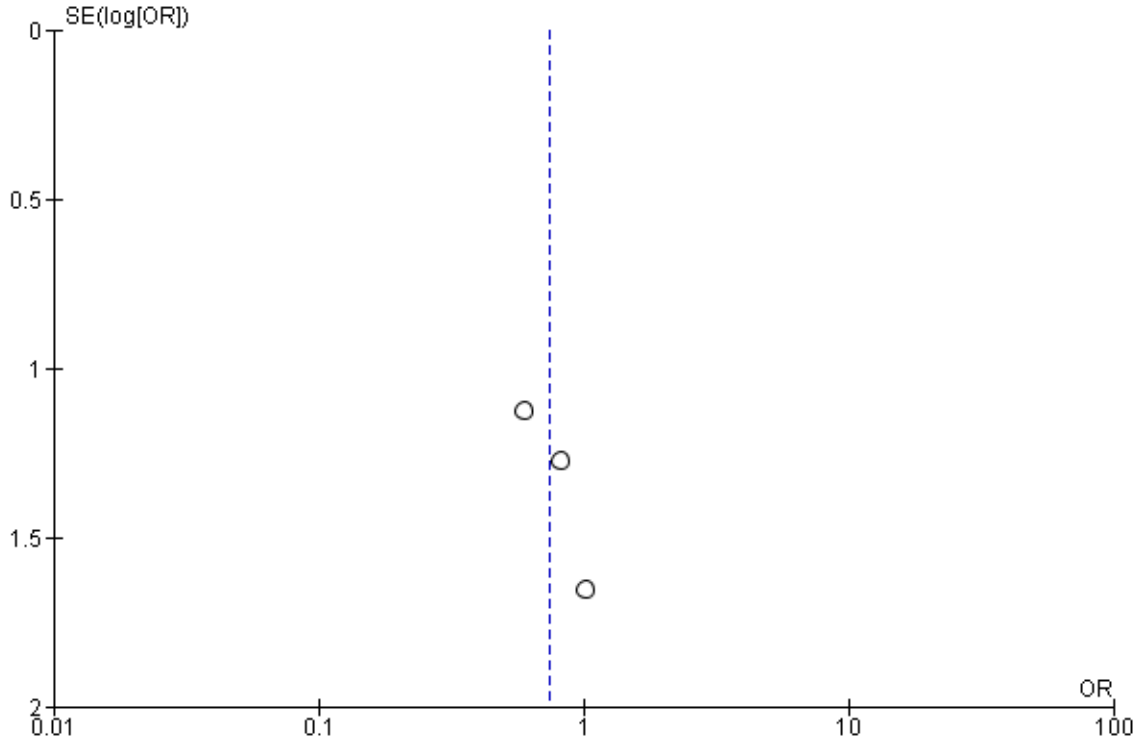
Supplementary Figure 100: Forest plot for *HLA-DQB106:04**



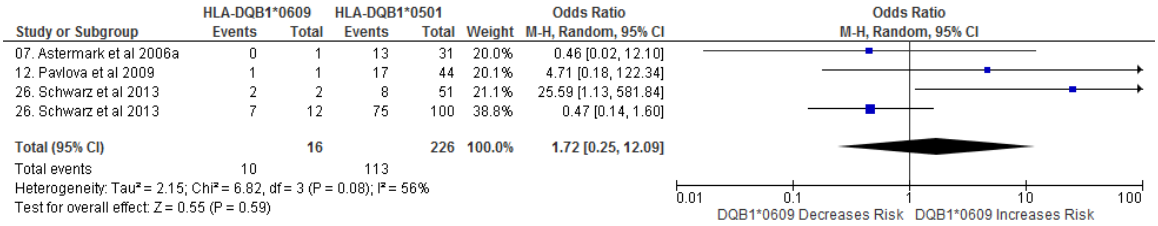
Supplementary Figure 101: Funnel plot for *HLA-DQB106:04**



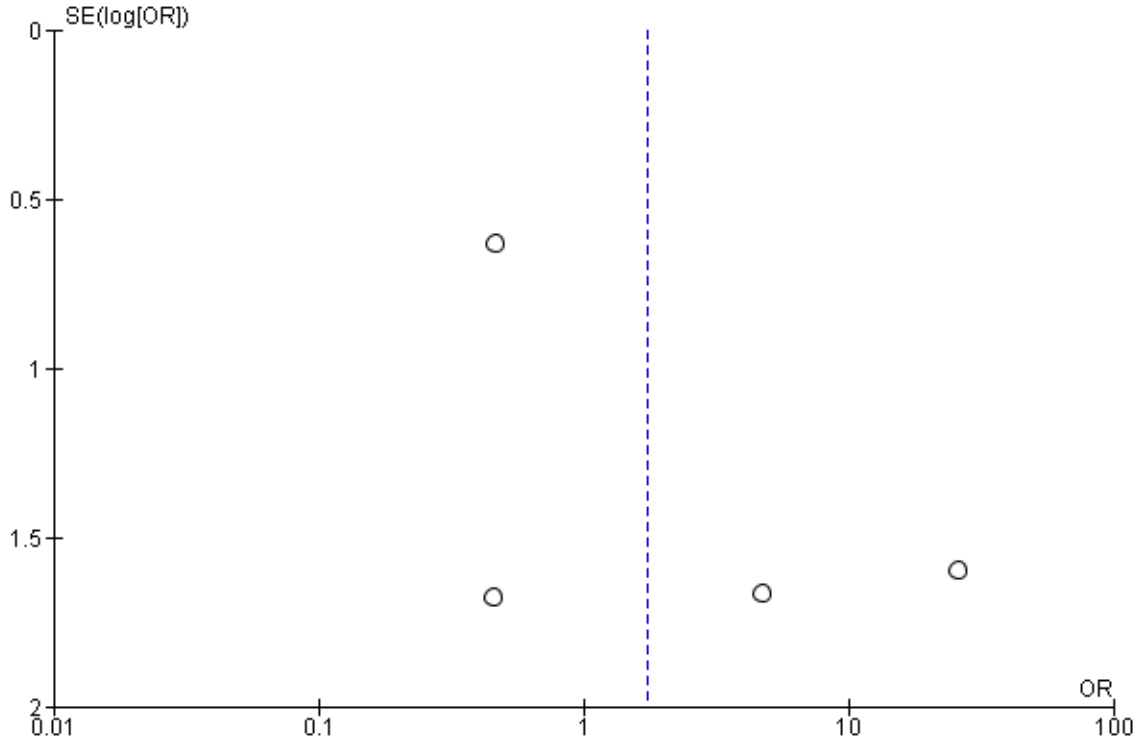
Supplementary Figure 102: Forest plot for *HLA-DQB106:05***



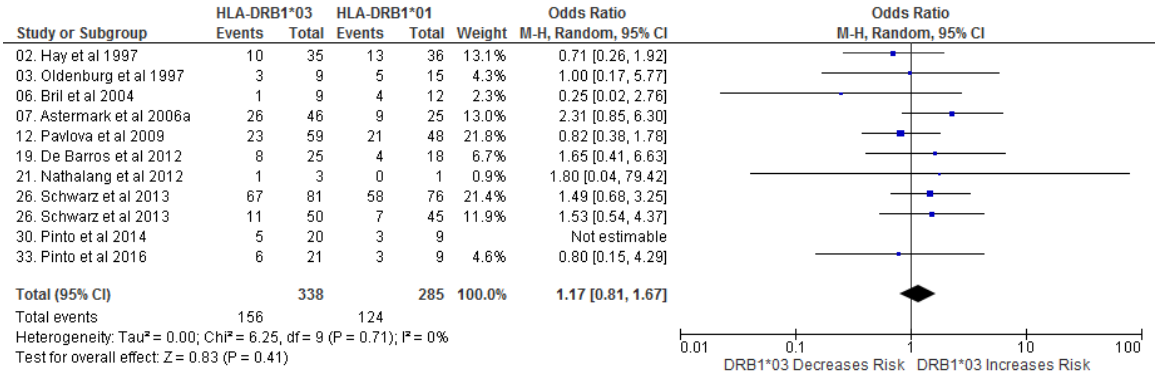
Supplementary Figure 103: Funnel plot for *HLA-DQB106:05***



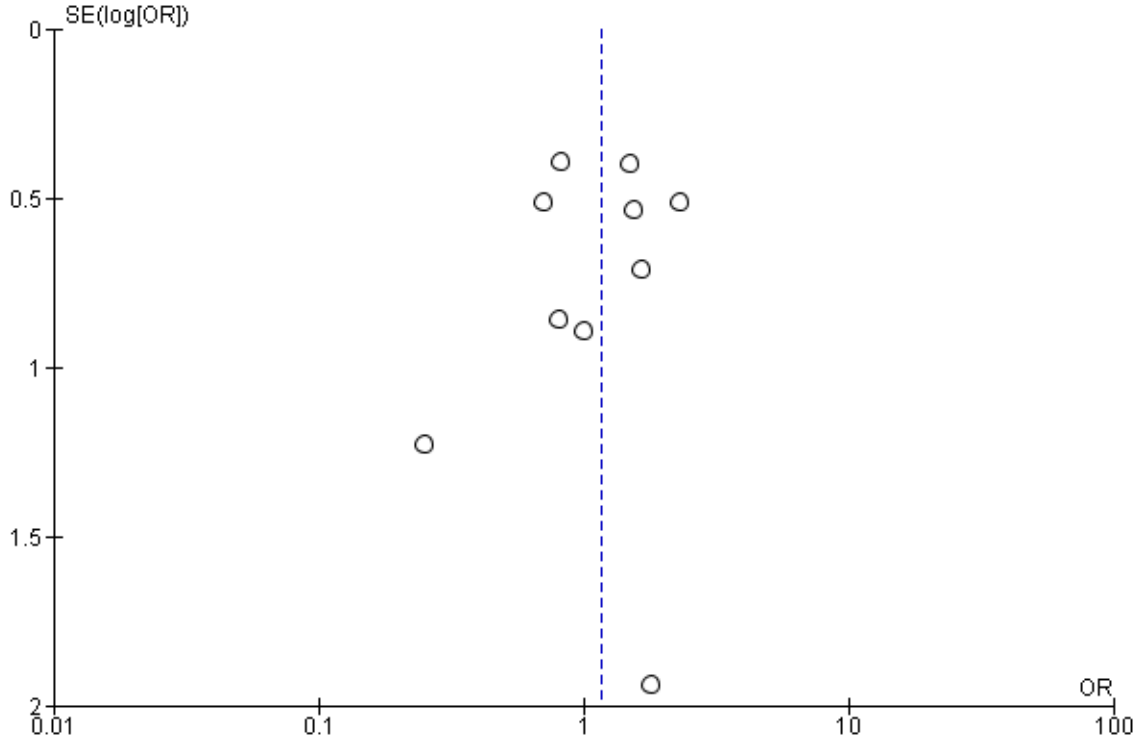
Supplementary Figure 104: Forest plot for *HLA-DQB106:09***



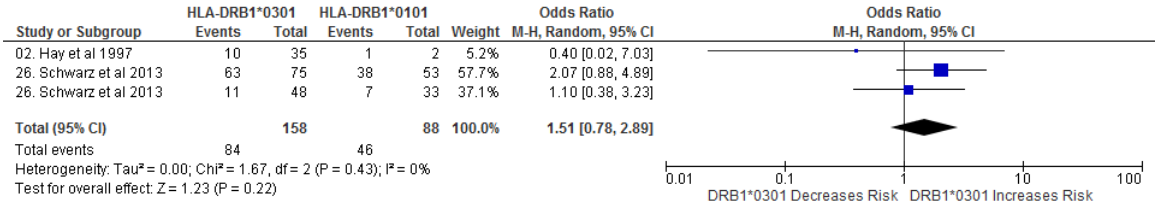
Supplementary Figure 105: Funnel plot for *HLA-DQB106:09***



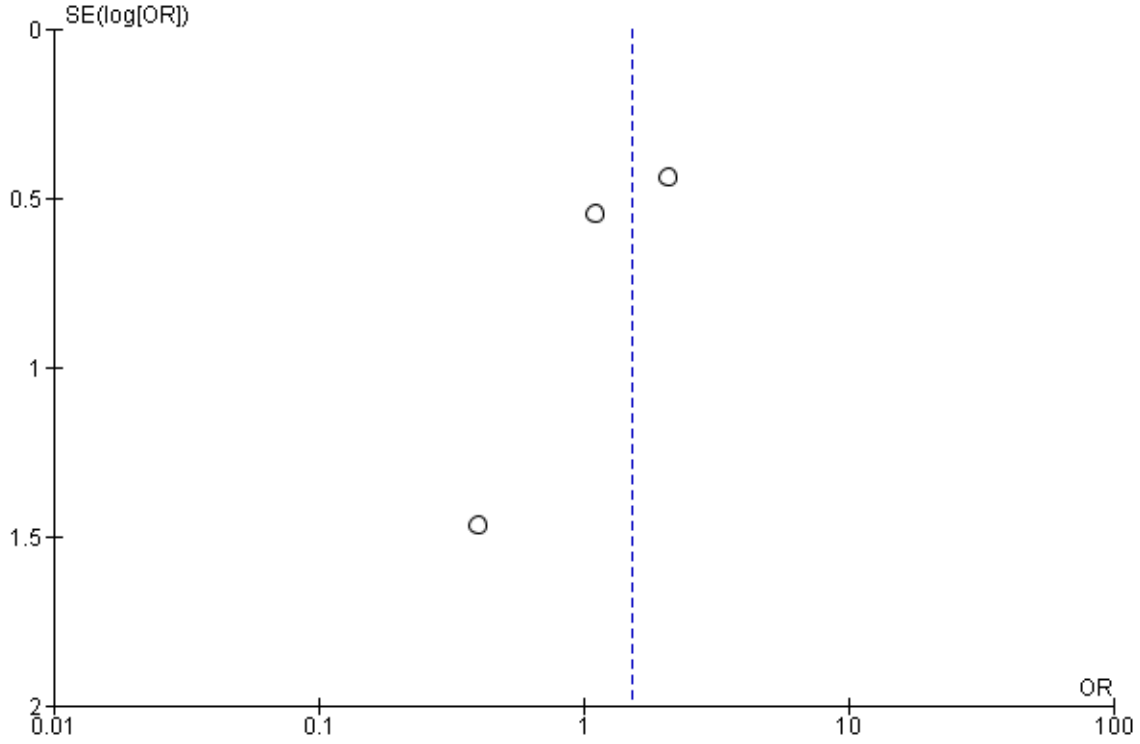
Supplementary Figure 106: Forest plot for *HLA-DRB103***



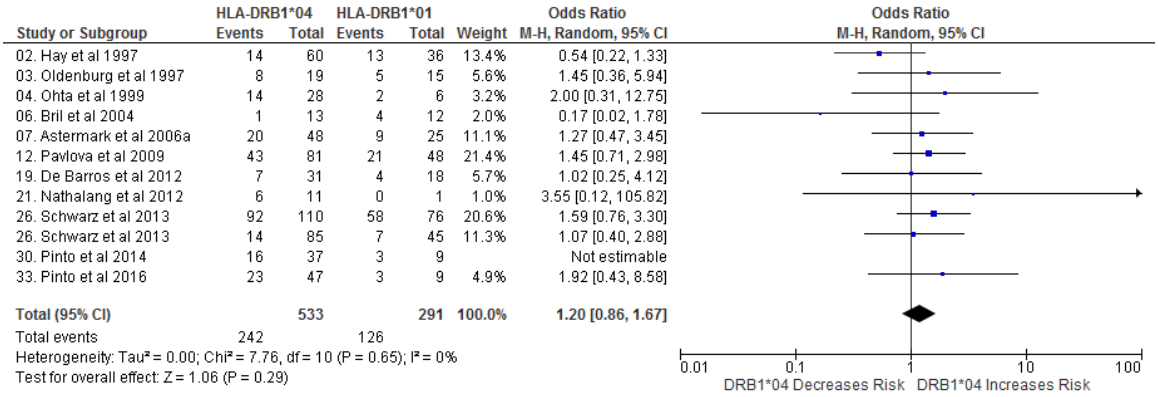
Supplementary Figure 107: Funnel plot for *HLA-DRB103**



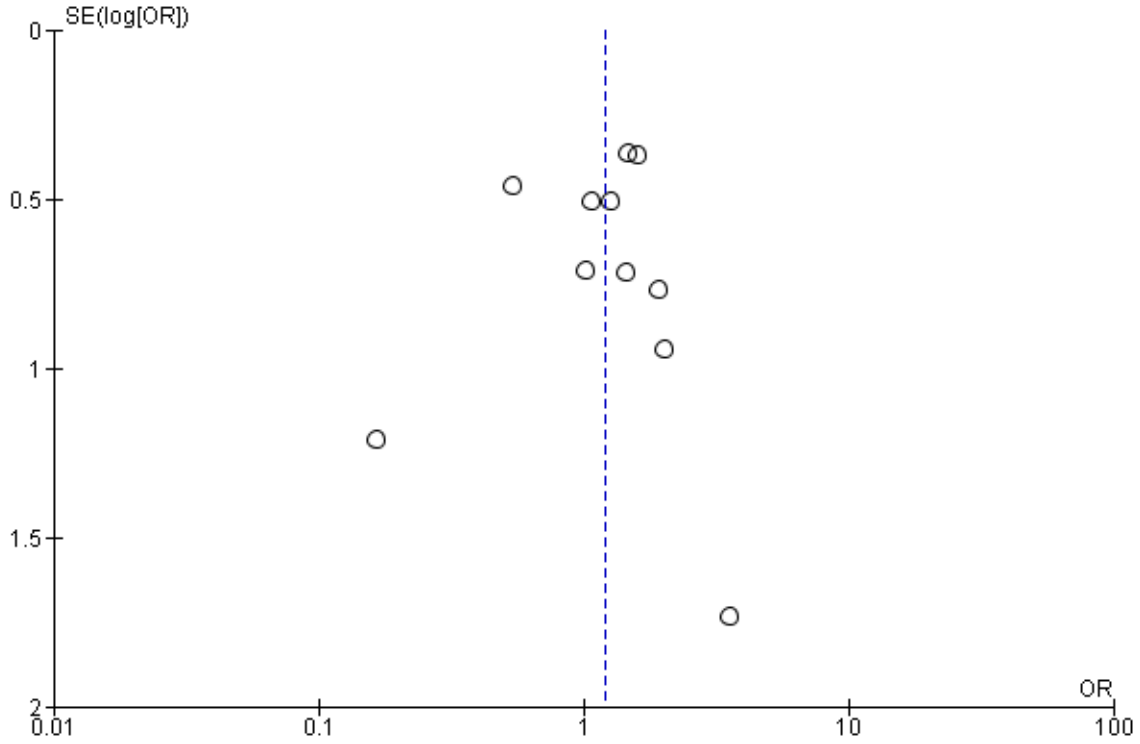
Supplementary Figure 108: Forest plot for *HLA-DRB103:01***



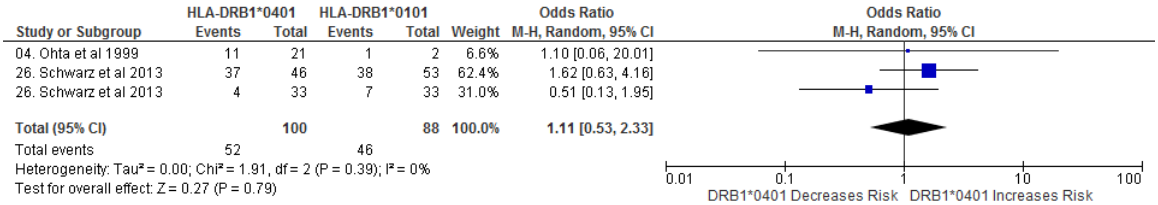
Supplementary Figure 109: Funnel plot for *HLA-DRB103:01***



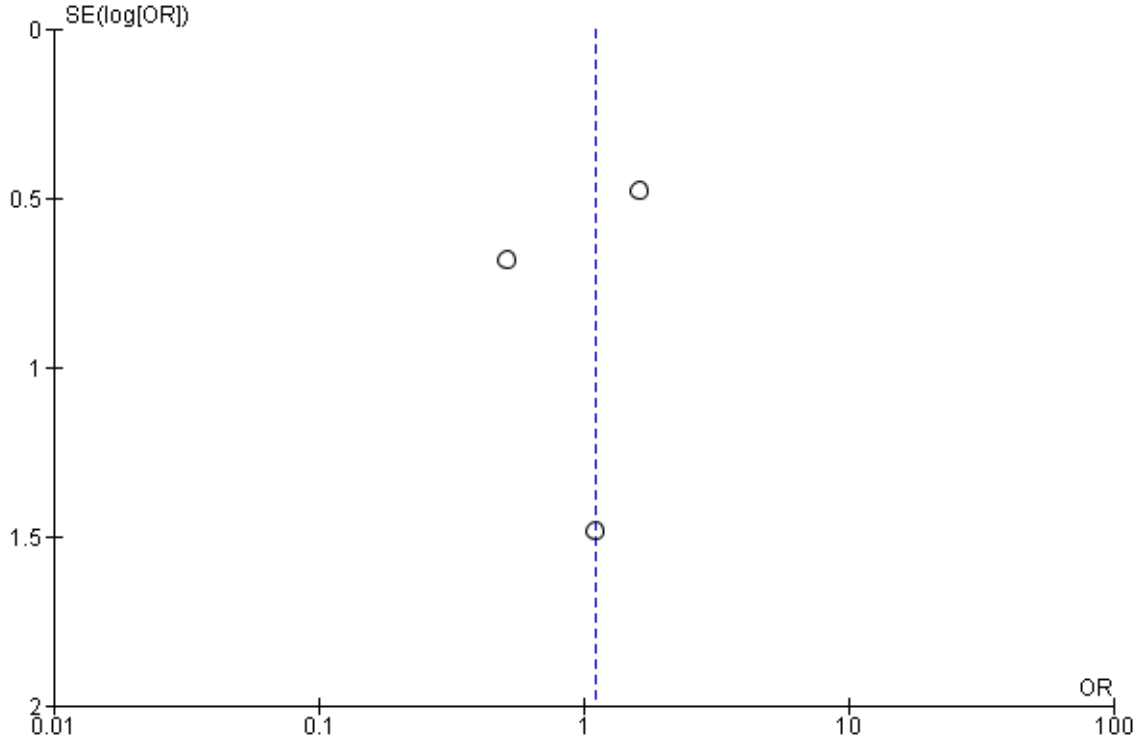
Supplementary Figure 110: Forest plot for *HLA-DRB104***



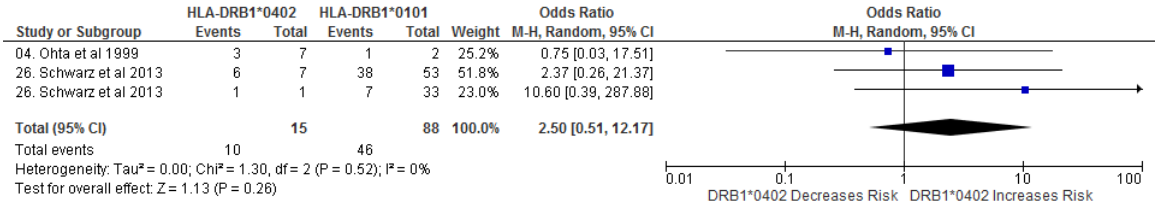
Supplementary Figure 111: Funnel plot for *HLA-DRB104**



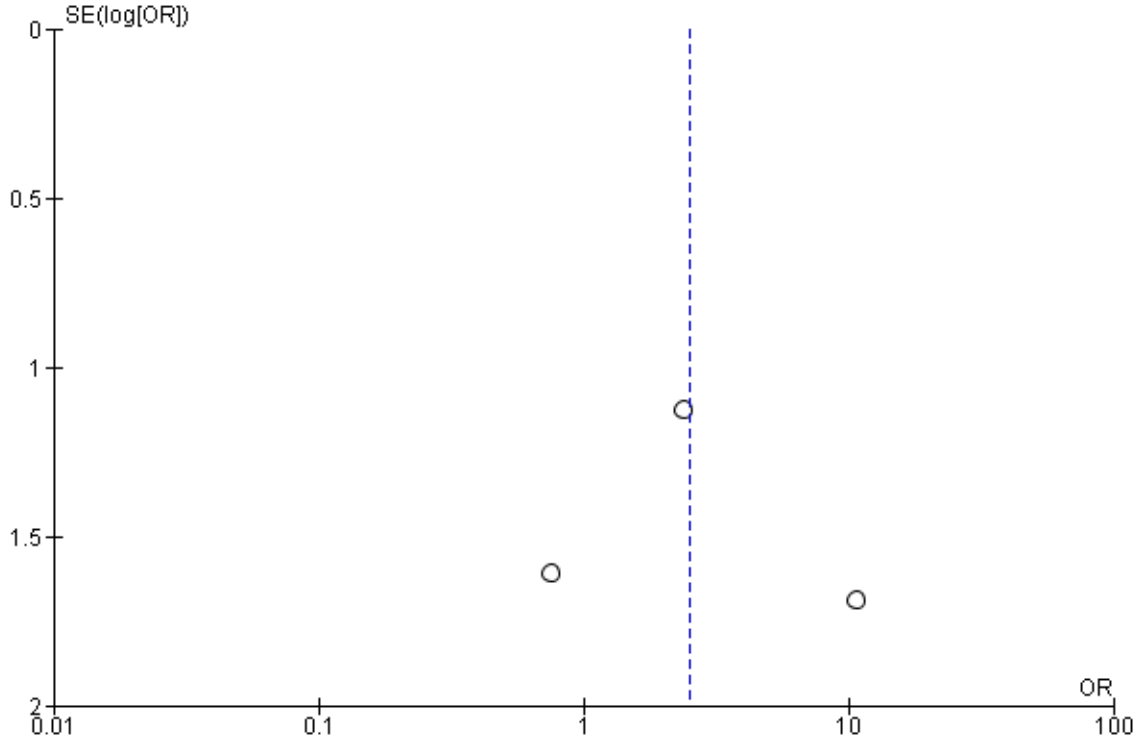
Supplementary Figure 112: Forest plot for *HLA-DRB1*04:01*



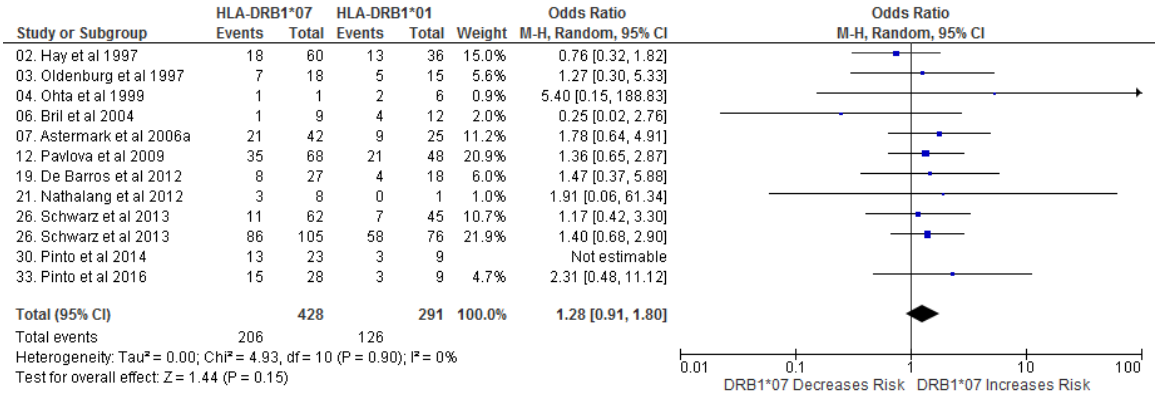
Supplementary Figure 113: Funnel plot for *HLA-DRB1*04:01*



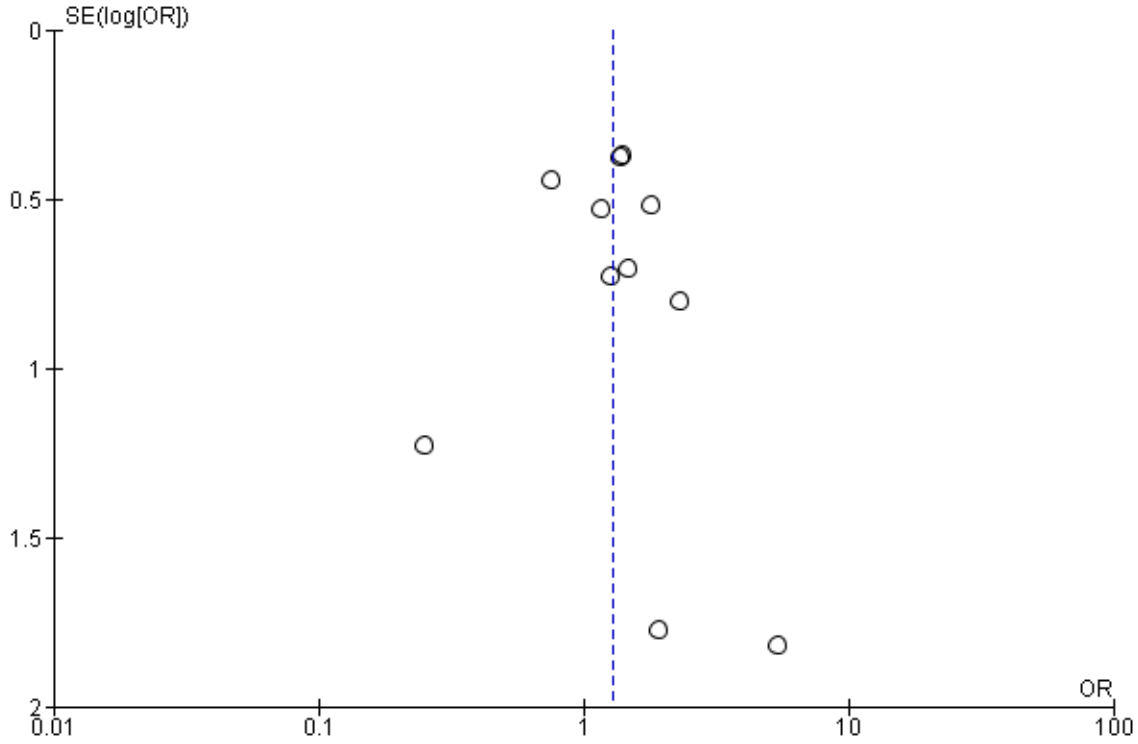
Supplementary Figure 114: Forest plot for *HLA-DRB104:02**



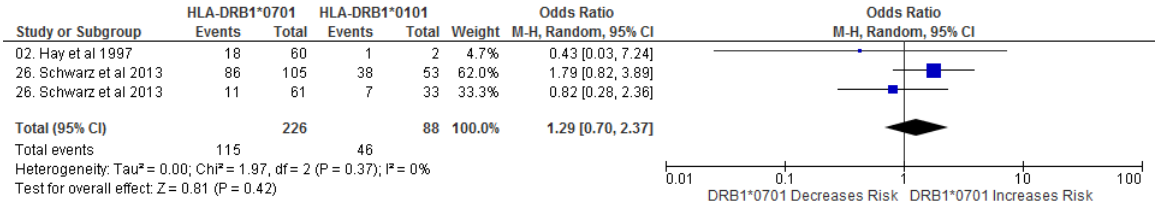
Supplementary Figure 115: Funnel plot for *HLA-DRB104:02**



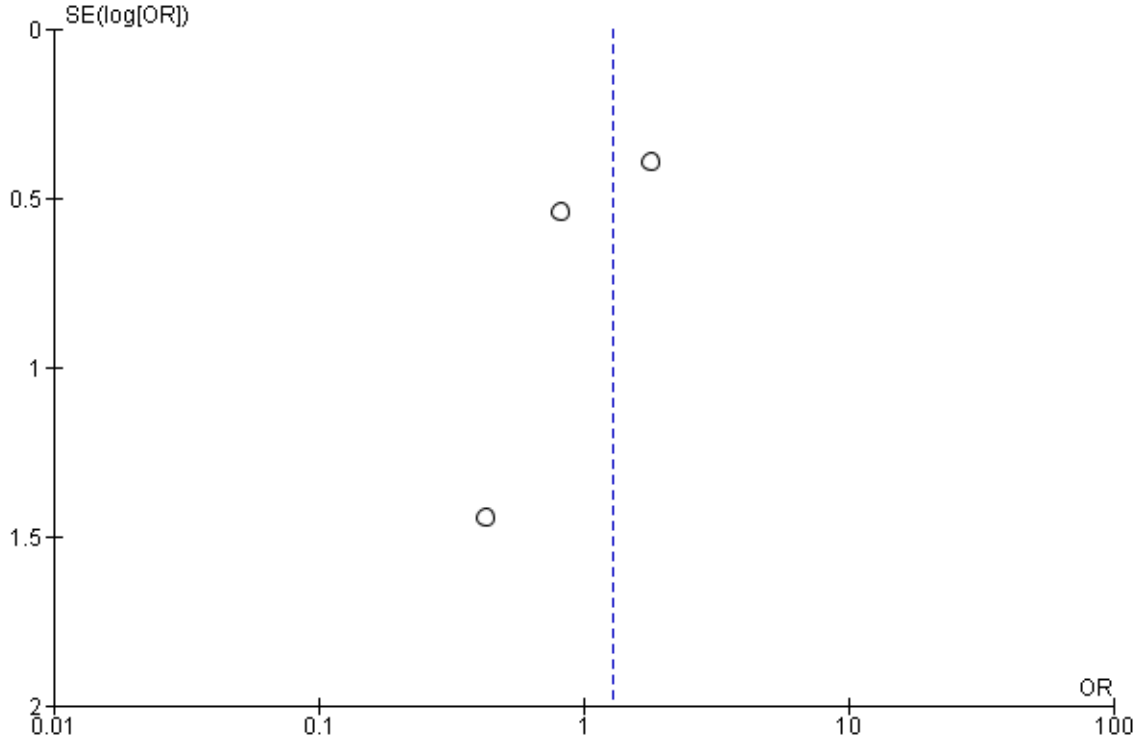
Supplementary Figure 116: Forest plot for *HLA-DRB1*07*



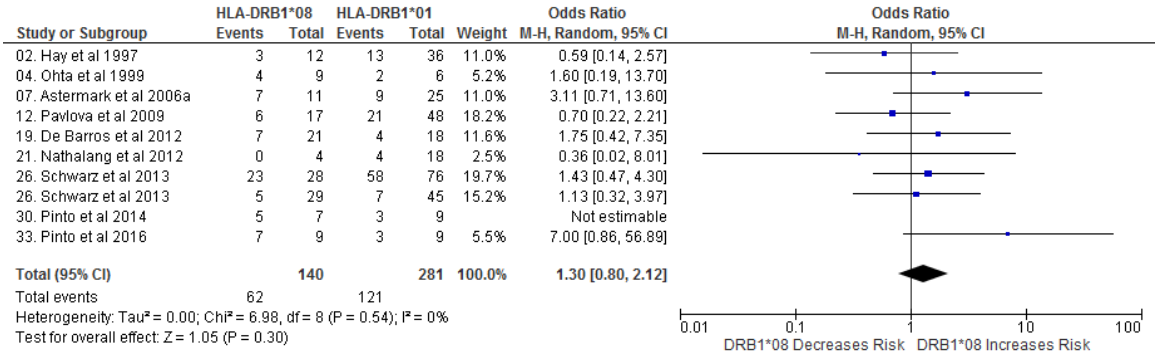
Supplementary Figure 117: Funnel plot for *HLA-DRB1*07*



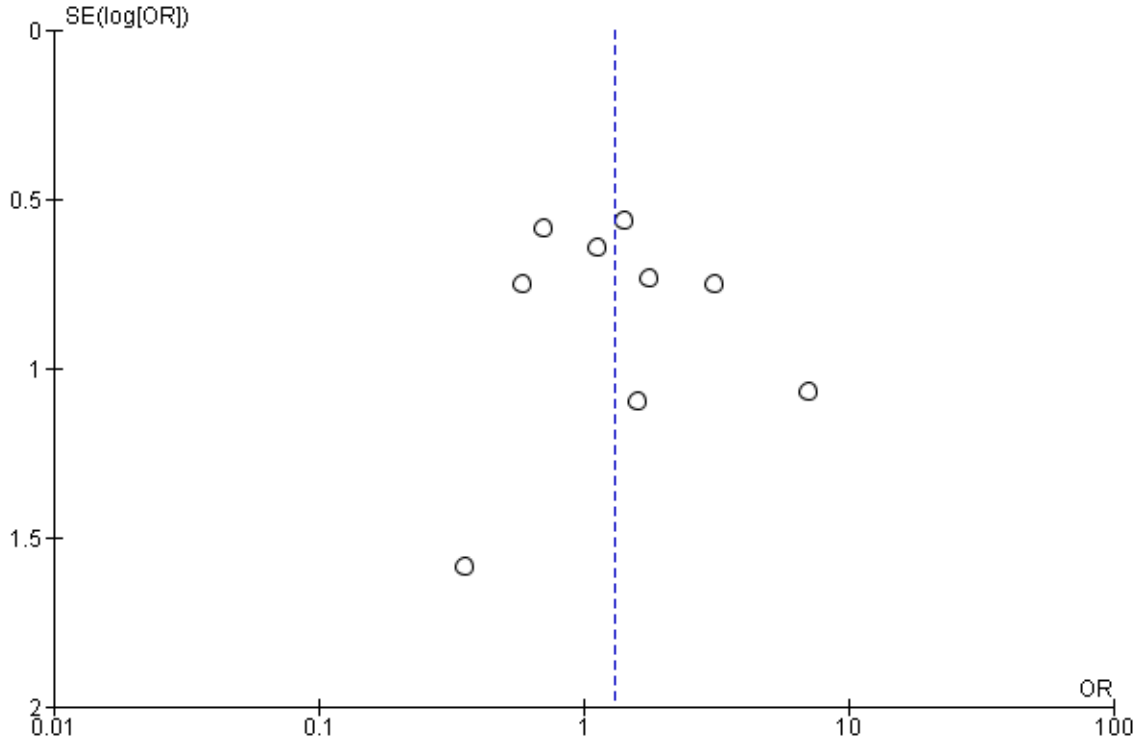
Supplementary Figure 118: Forest plot for *HLA-DRB1*07:01*



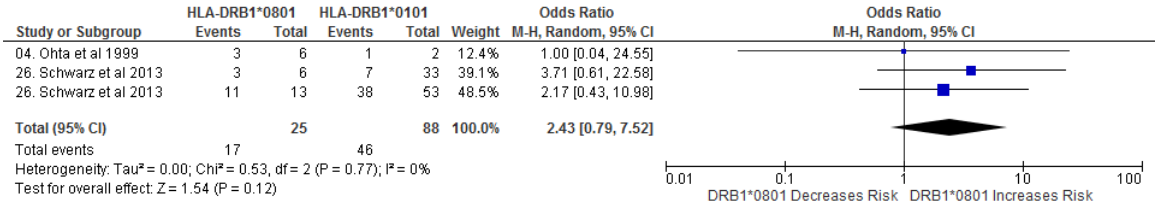
Supplementary Figure 119: Funnel plot for *HLA-DRB1*07:01*



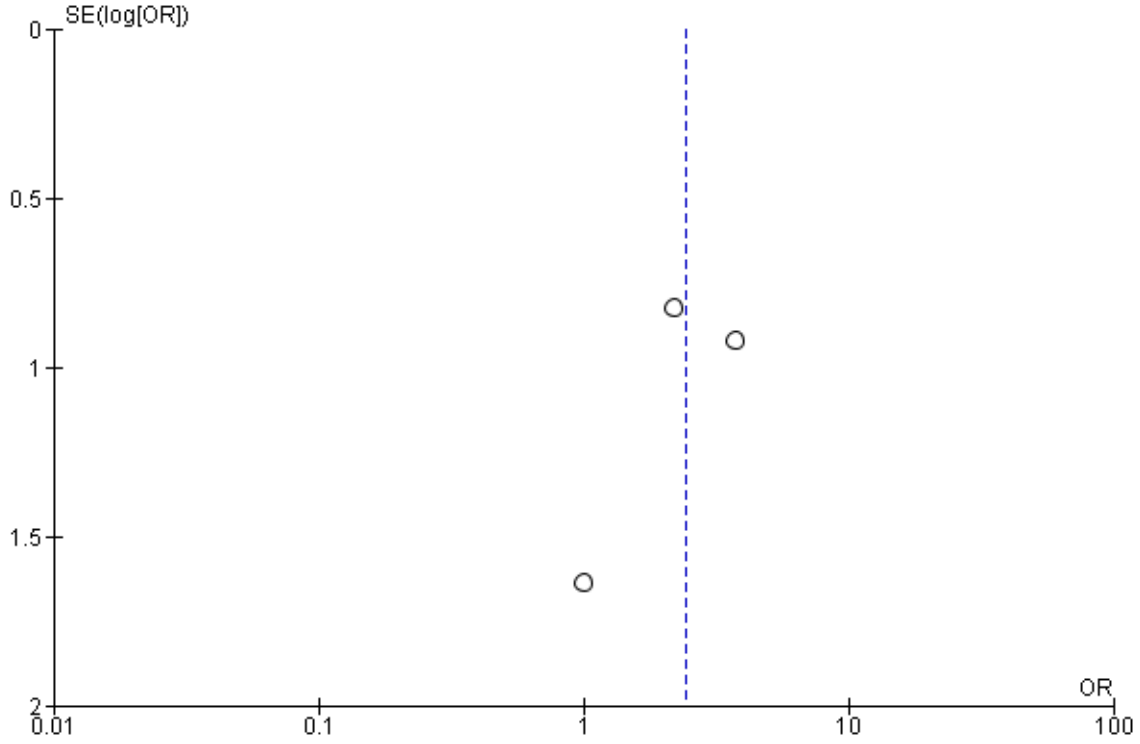
Supplementary Figure 120: Forest plot for *HLA-DRB108**



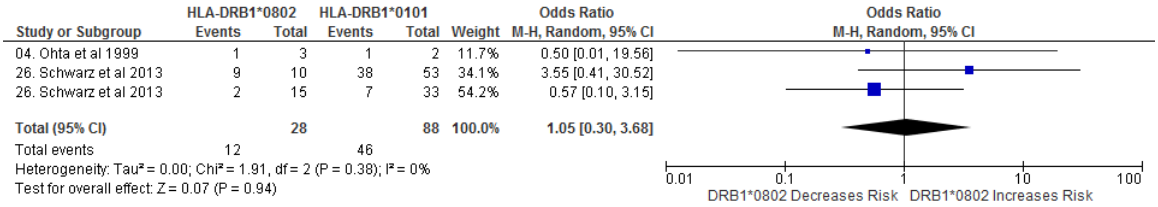
Supplementary Figure 121: Funnel plot for *HLA-DRB108**



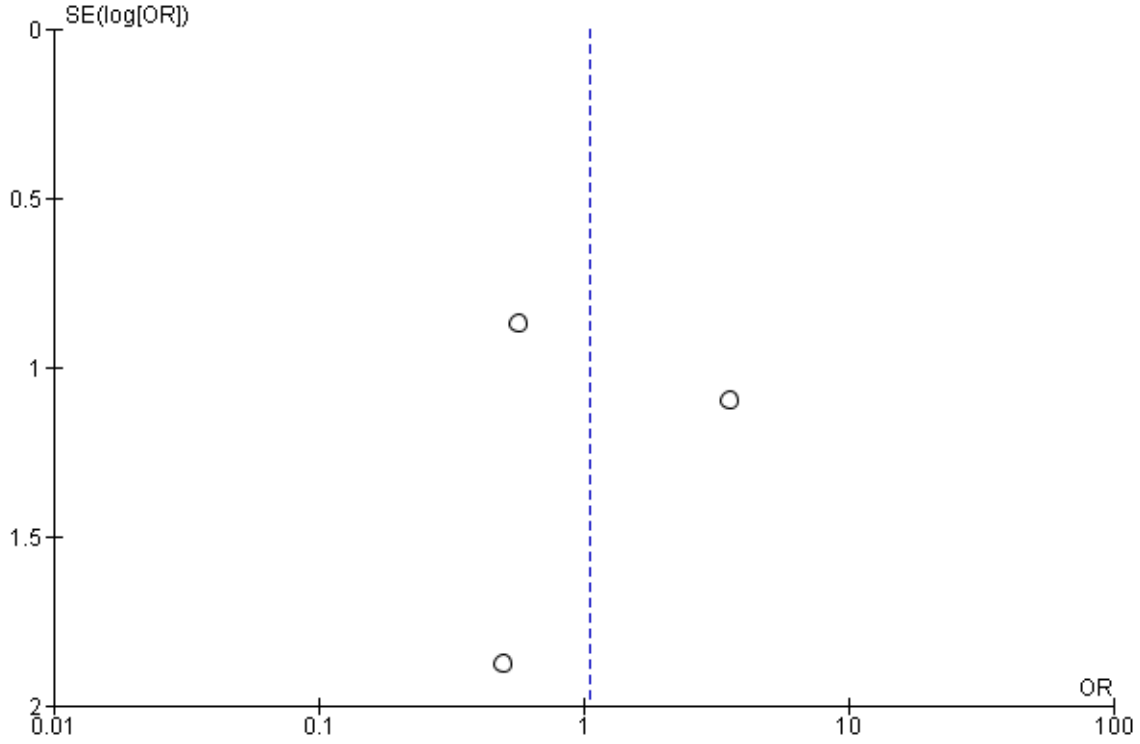
Supplementary Figure 122: Forest plot for *HLA-DRB1*08:01*



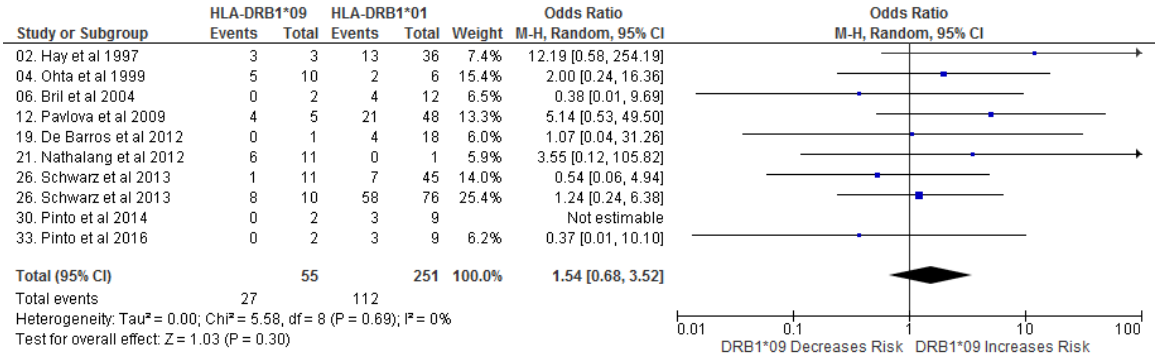
Supplementary Figure 123: Funnel plot for *HLA-DRB1*08:01*



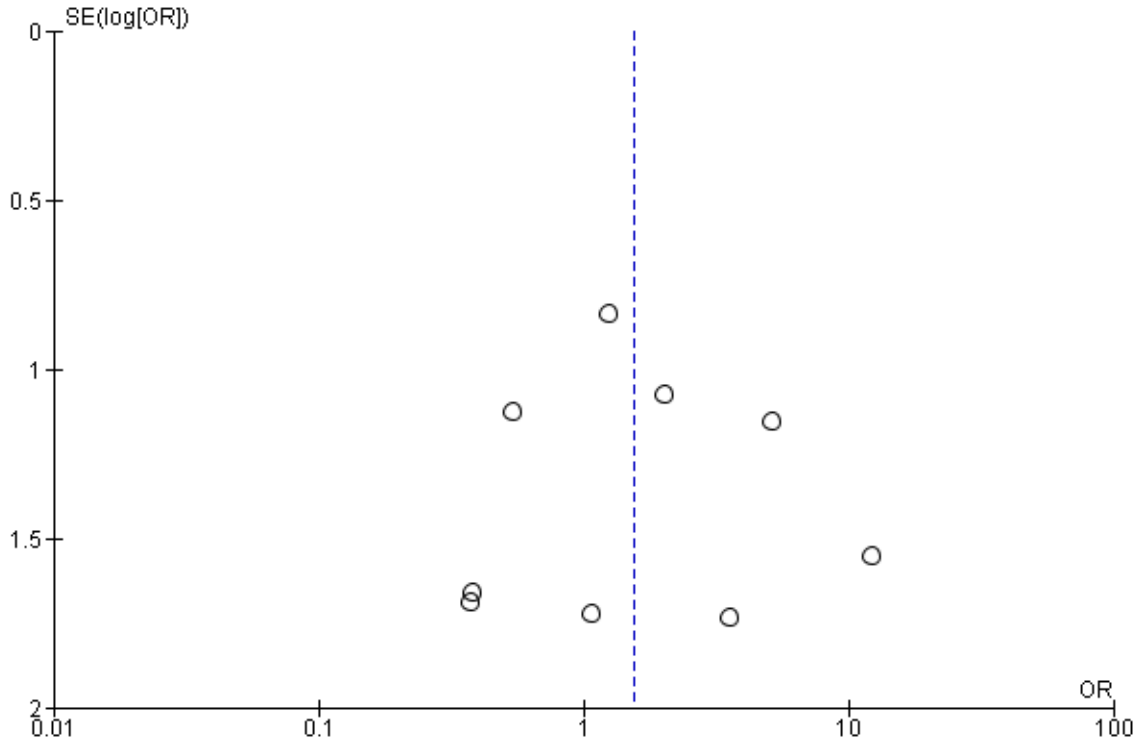
Supplementary Figure 124: Forest plot for *HLA-DRB108:02***



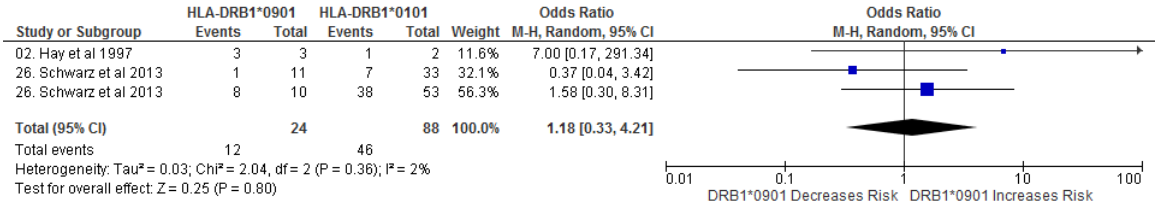
Supplementary Figure 125: Funnel plot for *HLA-DRB108:02***



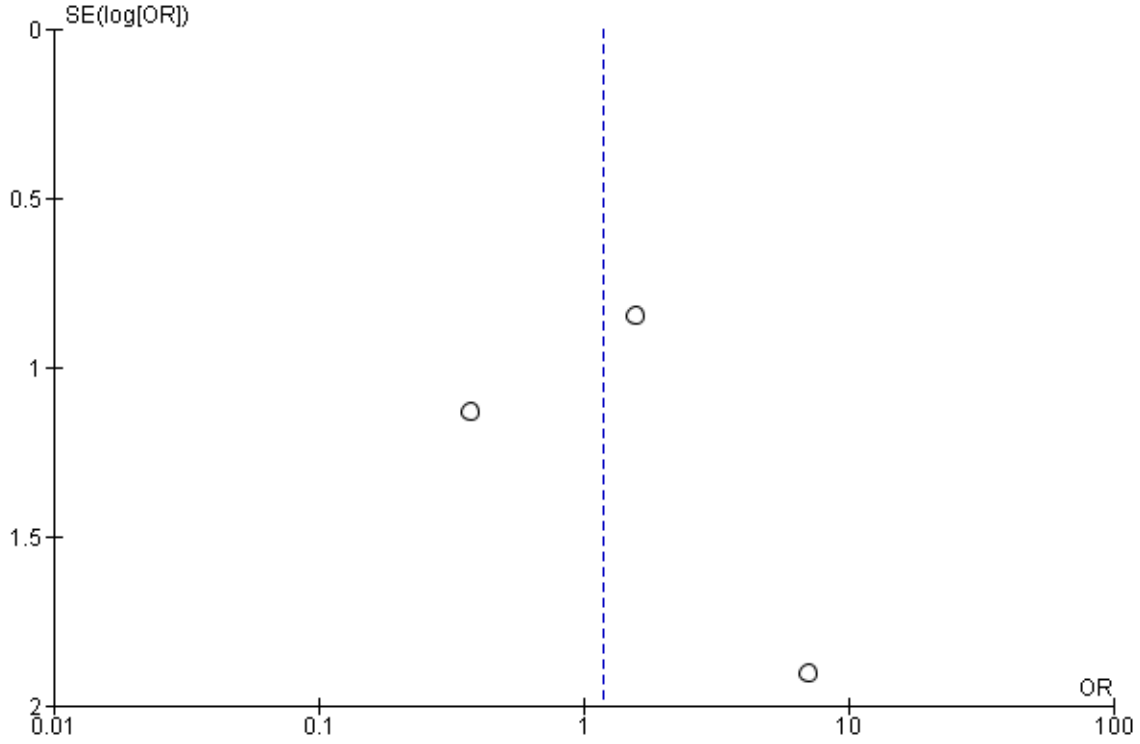
Supplementary Figure 126: Forest plot for *HLA-DRB109**



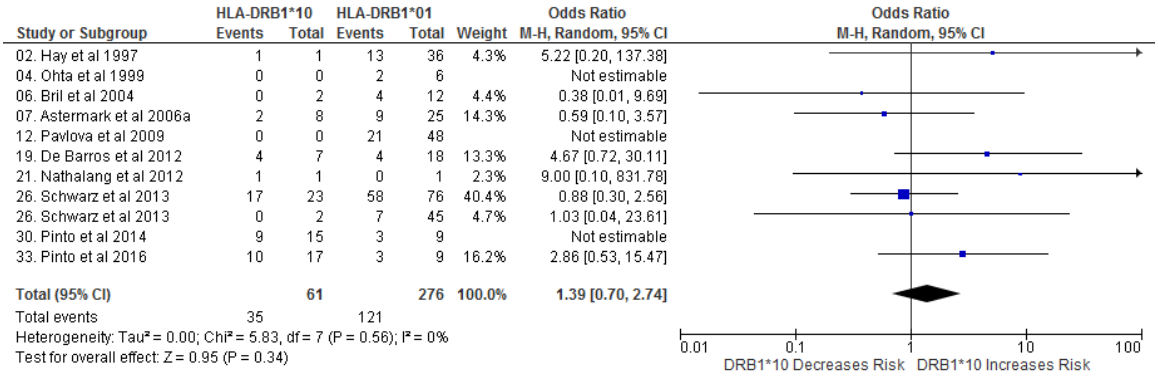
Supplementary Figure 127: Funnel plot for *HLA-DRB109**



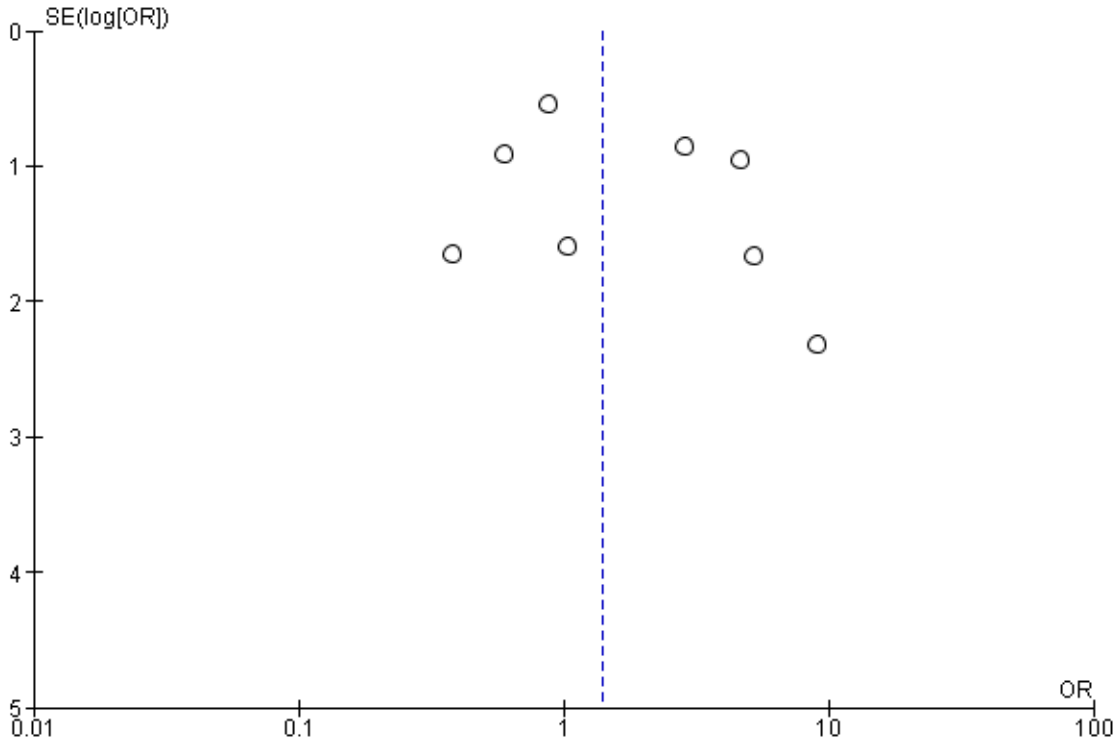
Supplementary Figure 128: Forest plot for *HLA-DRB1*09:01*



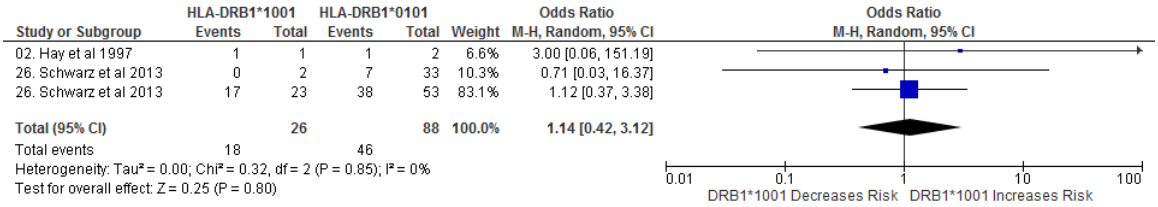
Supplementary Figure 129: Funnel plot for *HLA-DRB1*09:01*



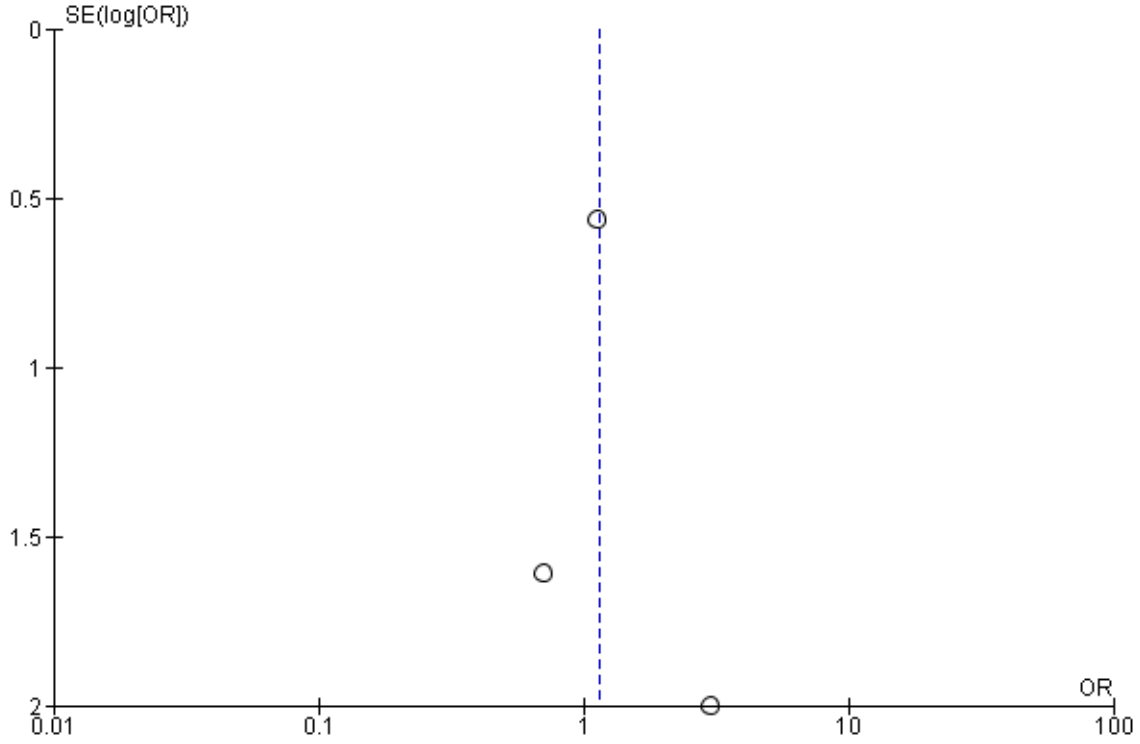
Supplementary Figure 130: Forest plot for *HLA-DRB110**



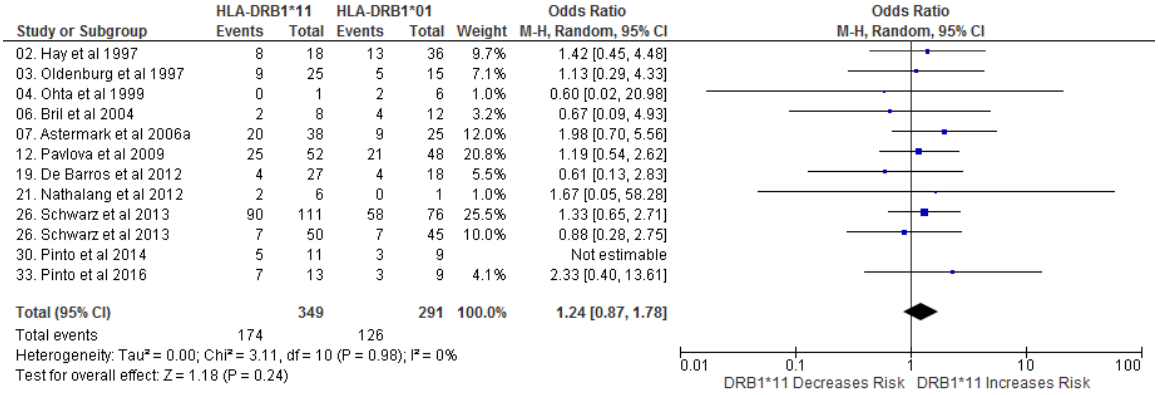
Supplementary Figure 131: Funnel plot for *HLA-DRB110**



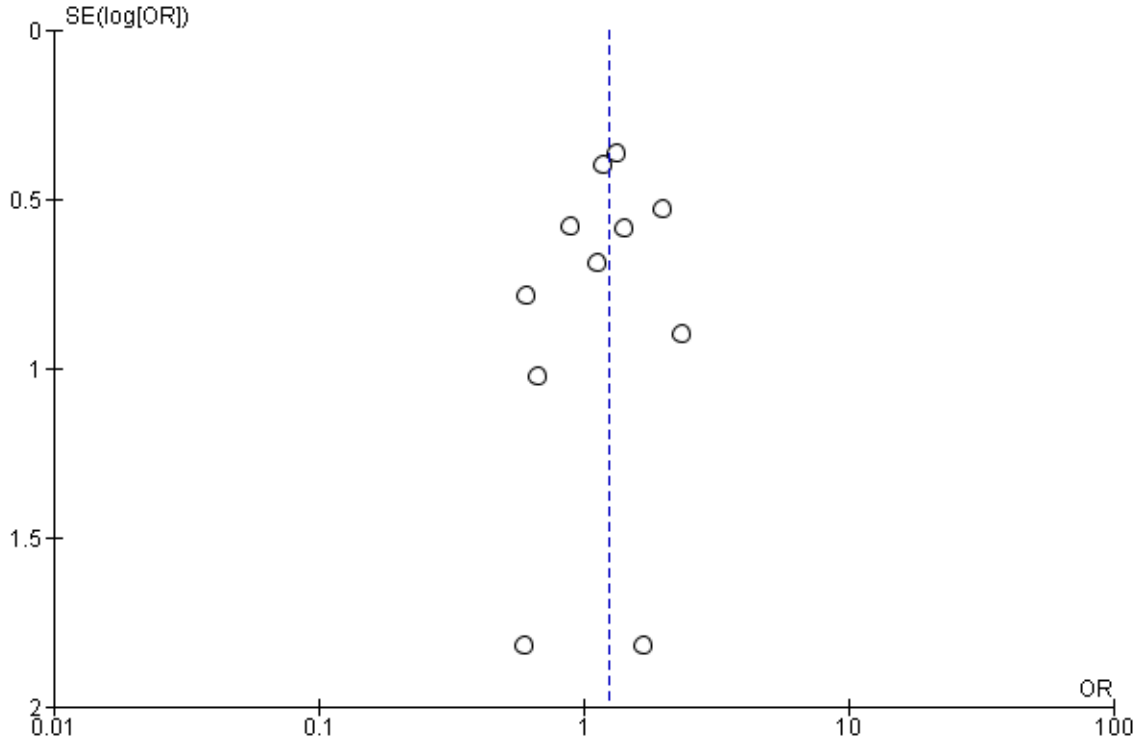
Supplementary Figure 132: Forest plot for *HLA-DRB1*10:01*



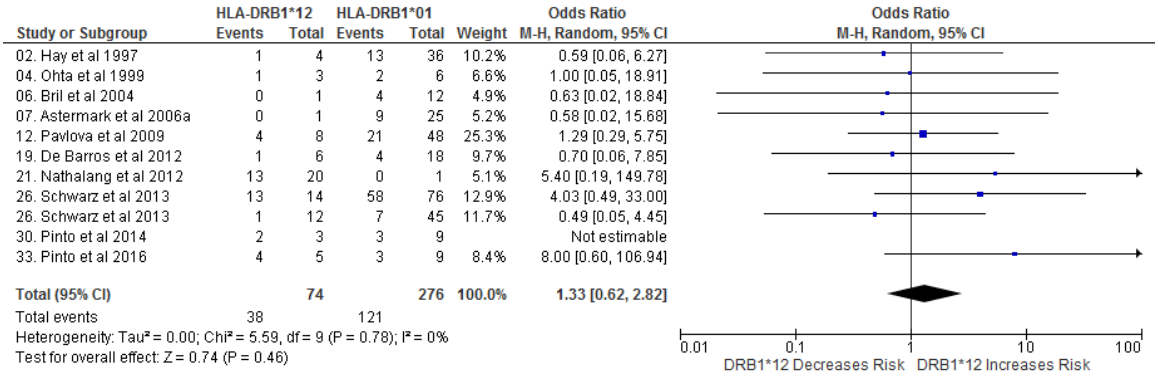
Supplementary Figure 133: Funnel plot for *HLA-DRB1*10:01*



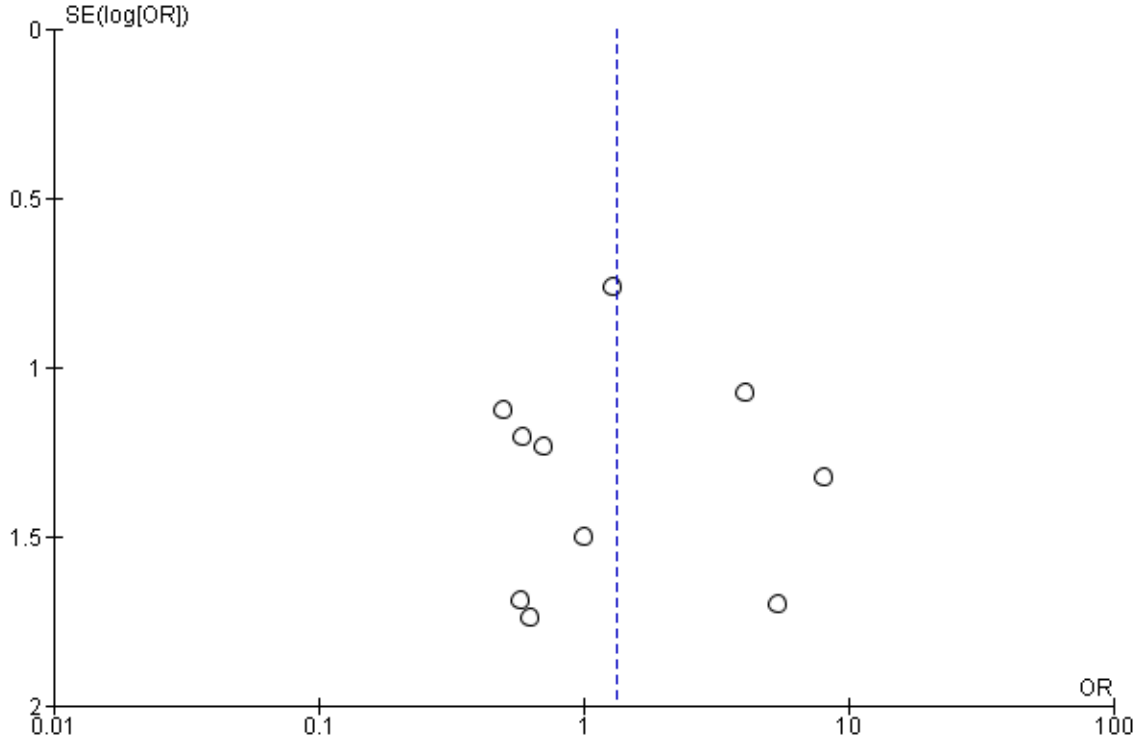
Supplementary Figure 134: Forest plot for *HLA-DRB111**



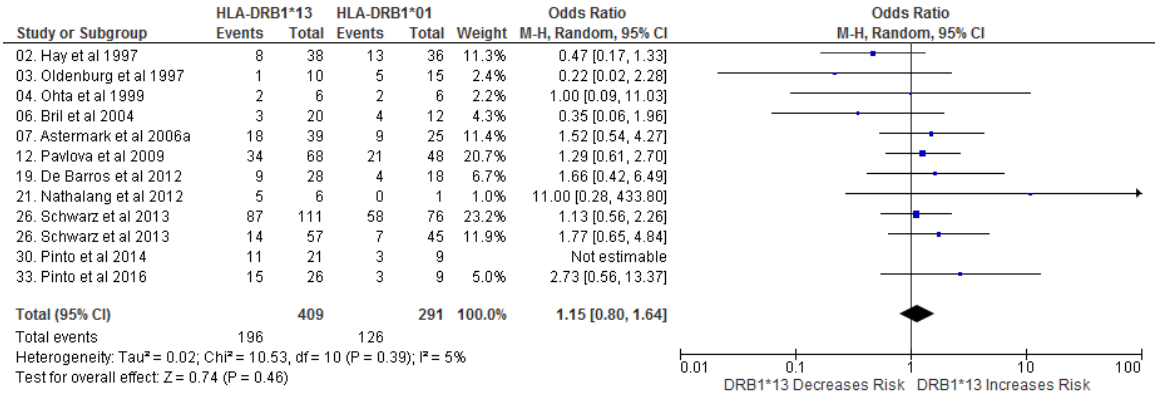
Supplementary Figure 135: Funnel plot for *HLA-DRB111**



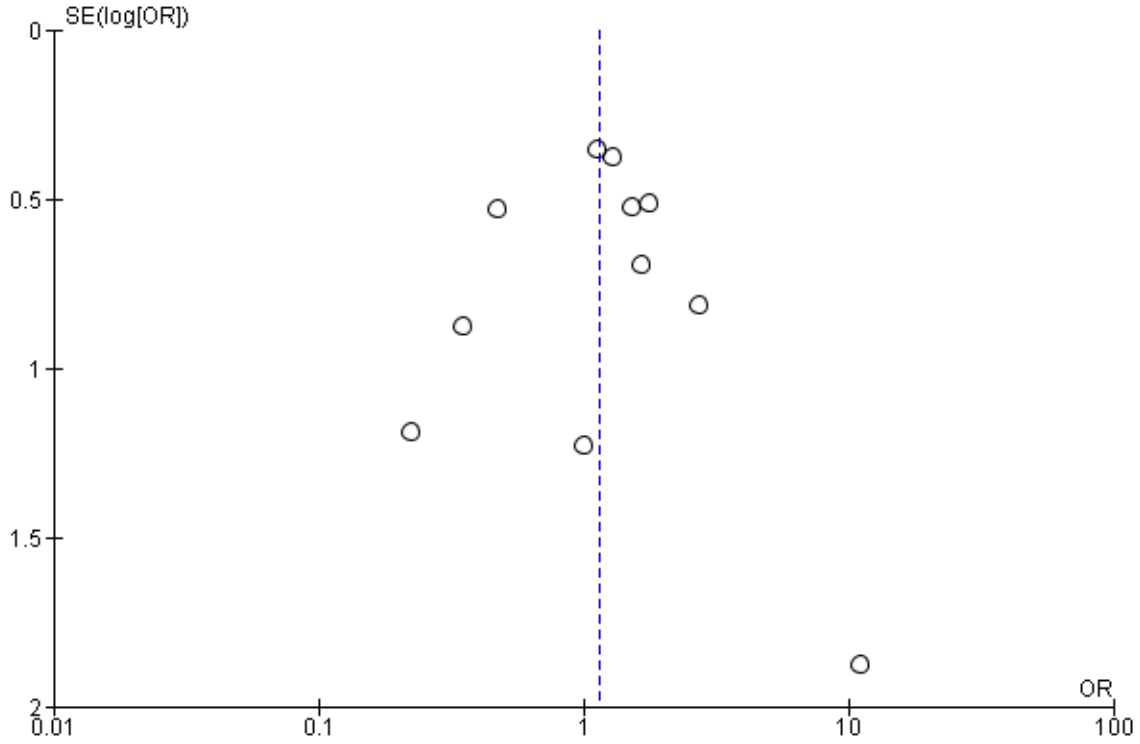
Supplementary Figure 136: Forest plot for *HLA-DRB1*12*



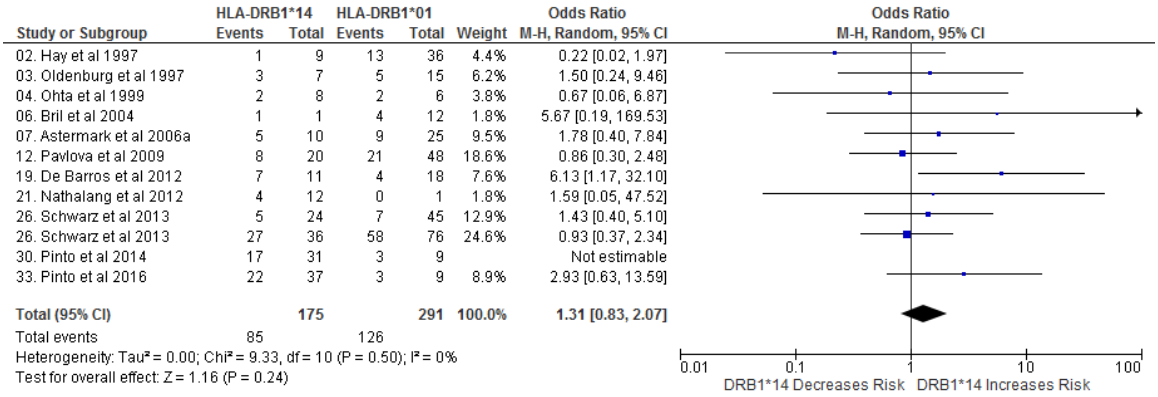
Supplementary Figure 137: Funnel plot for *HLA-DRB1*12*



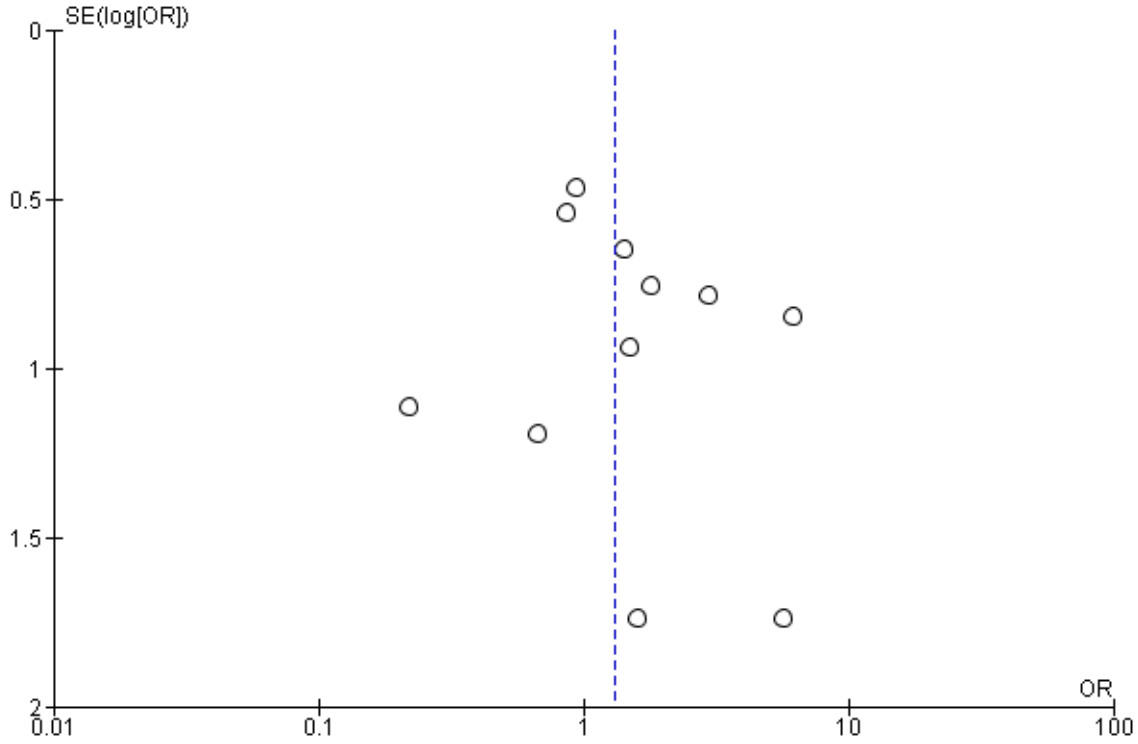
Supplementary Figure 138: Forest plot for *HLA-DRB113**



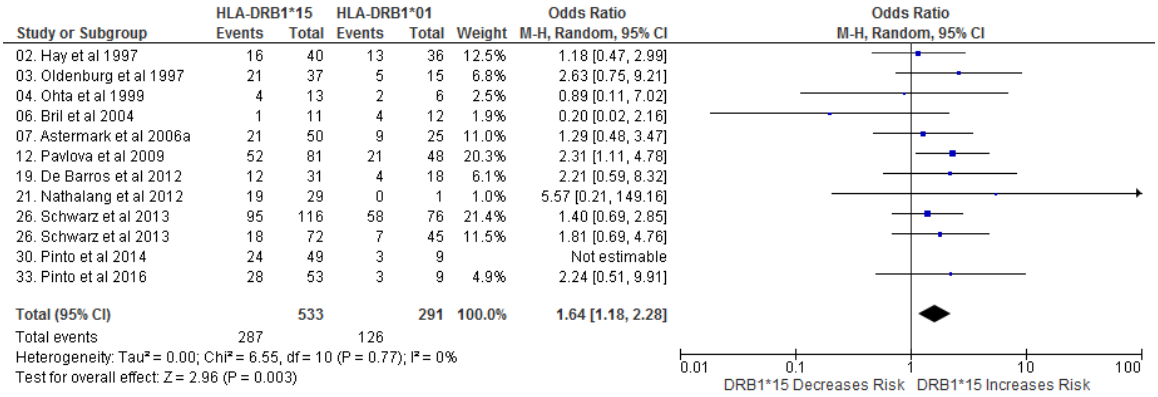
Supplementary Figure 139: Funnel plot for *HLA-DRB113**



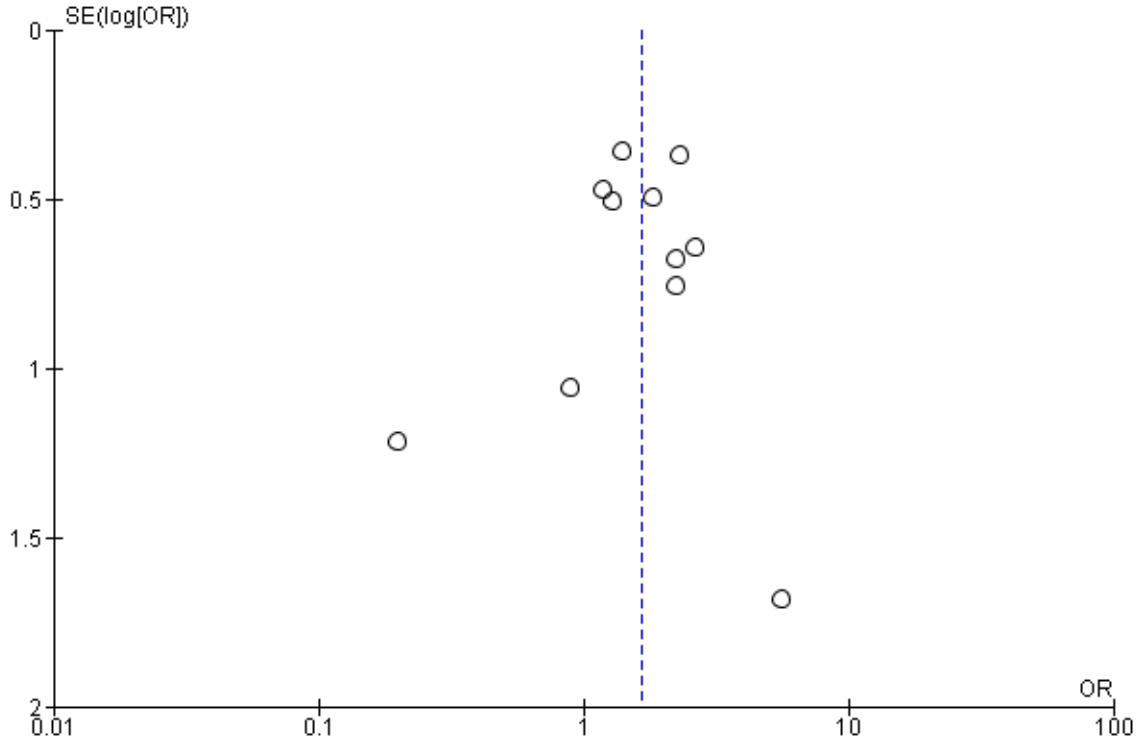
Supplementary Figure 140: Forest plot for *HLA-DRB114**



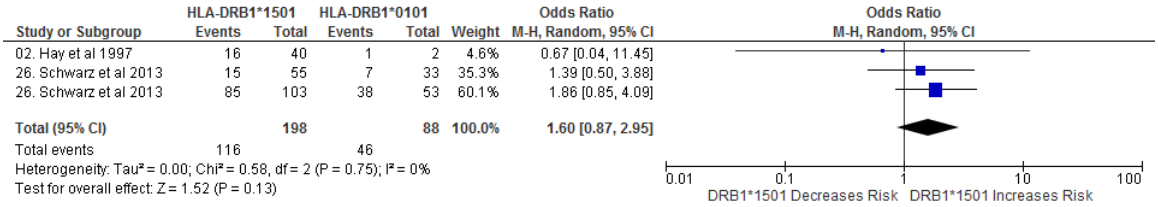
Supplementary Figure 141: Funnel plot for *HLA-DRB114**



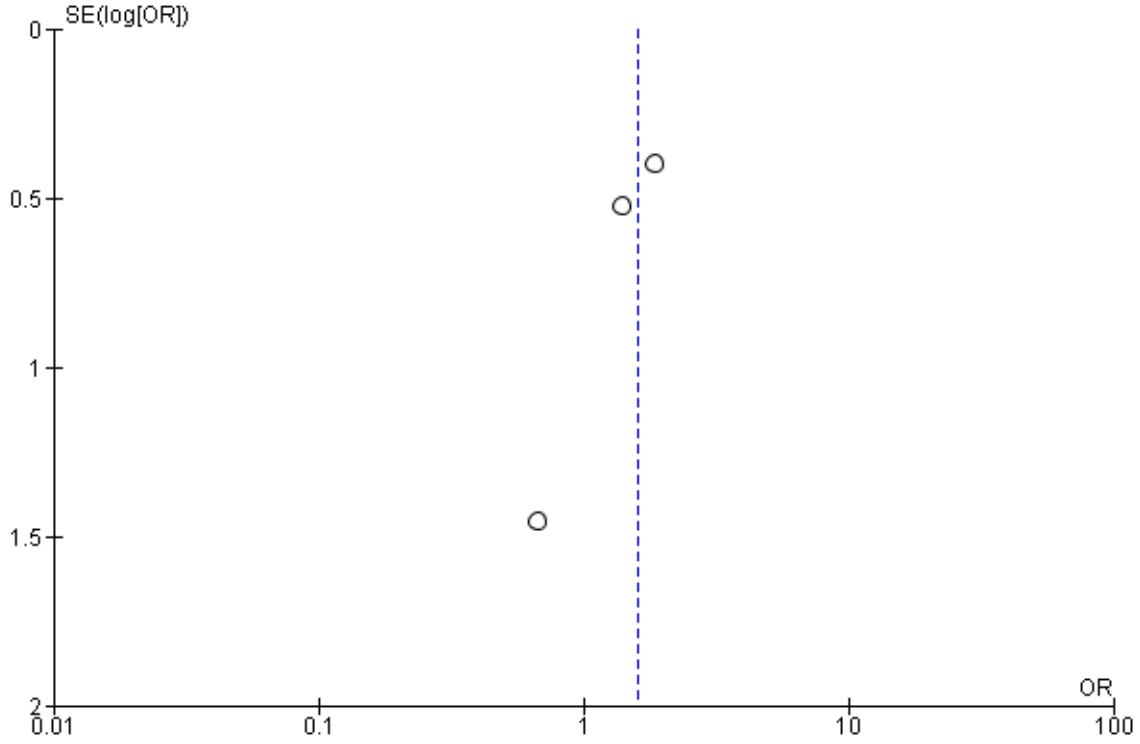
Supplementary Figure 142: Forest plot for *HLA-DRB115**



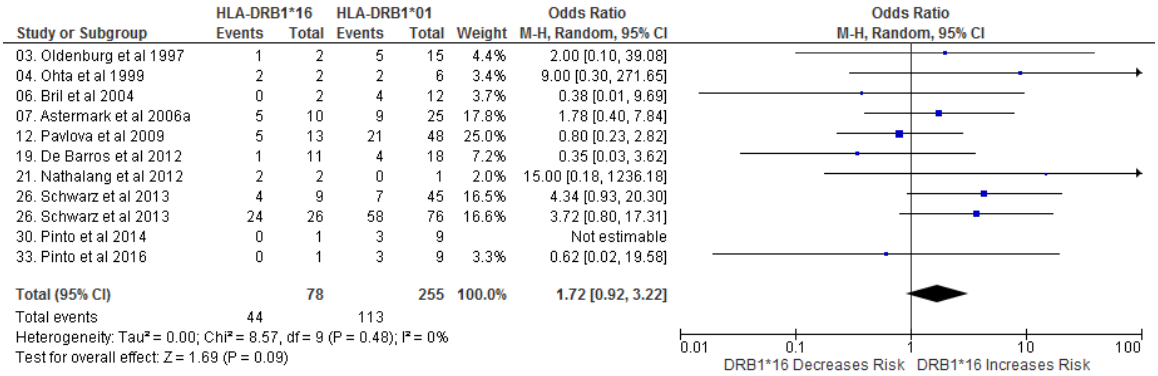
Supplementary Figure 143: Funnel plot for *HLA-DRB115**



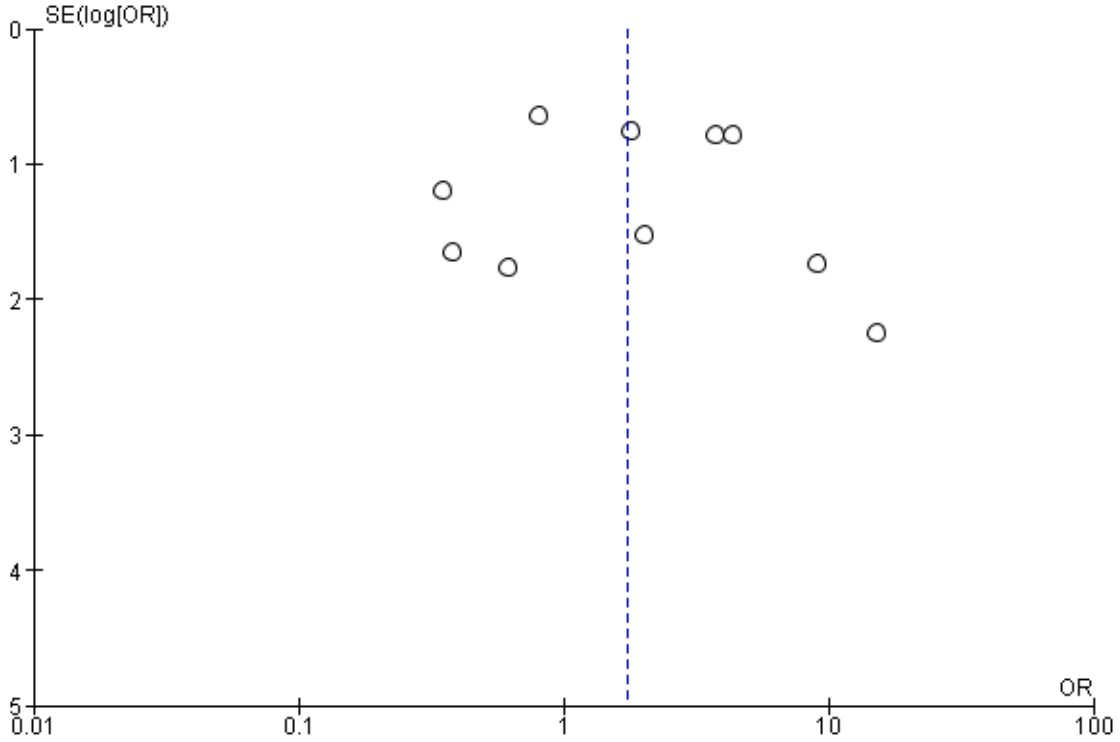
Supplementary Figure 144: Forest plot for *HLA-DRB1*15:01*



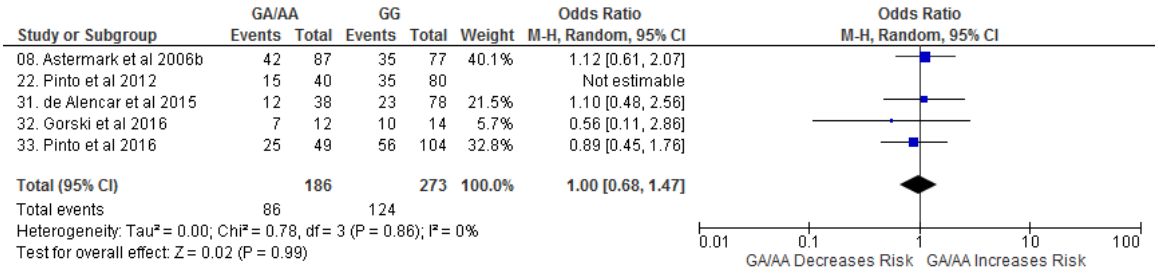
Supplementary Figure 145: Funnel plot for *HLA-DRB1*15:01*



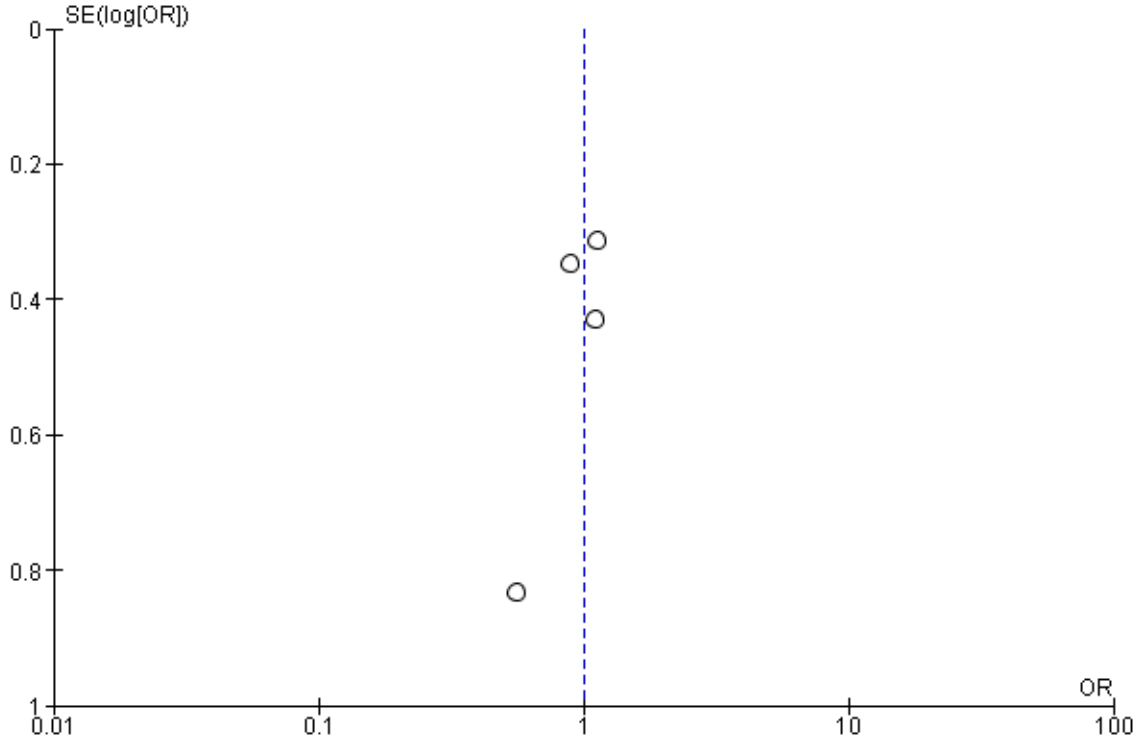
Supplementary Figure 146: Forest plot for *HLA-DRB116**



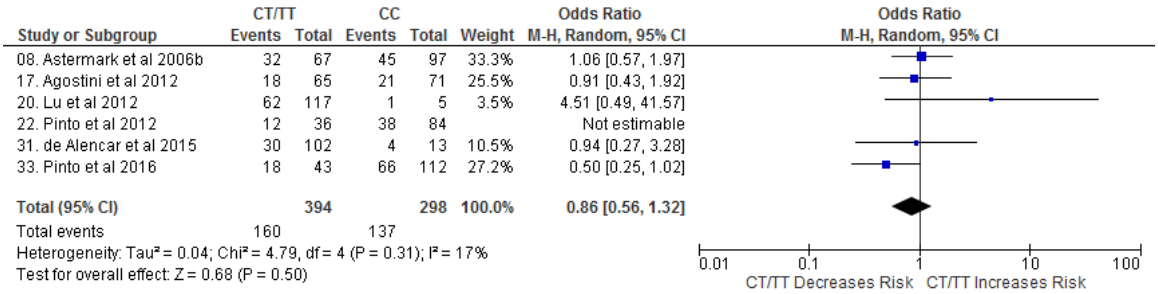
Supplementary Figure 147: Funnel plot for *HLA-DRB116**



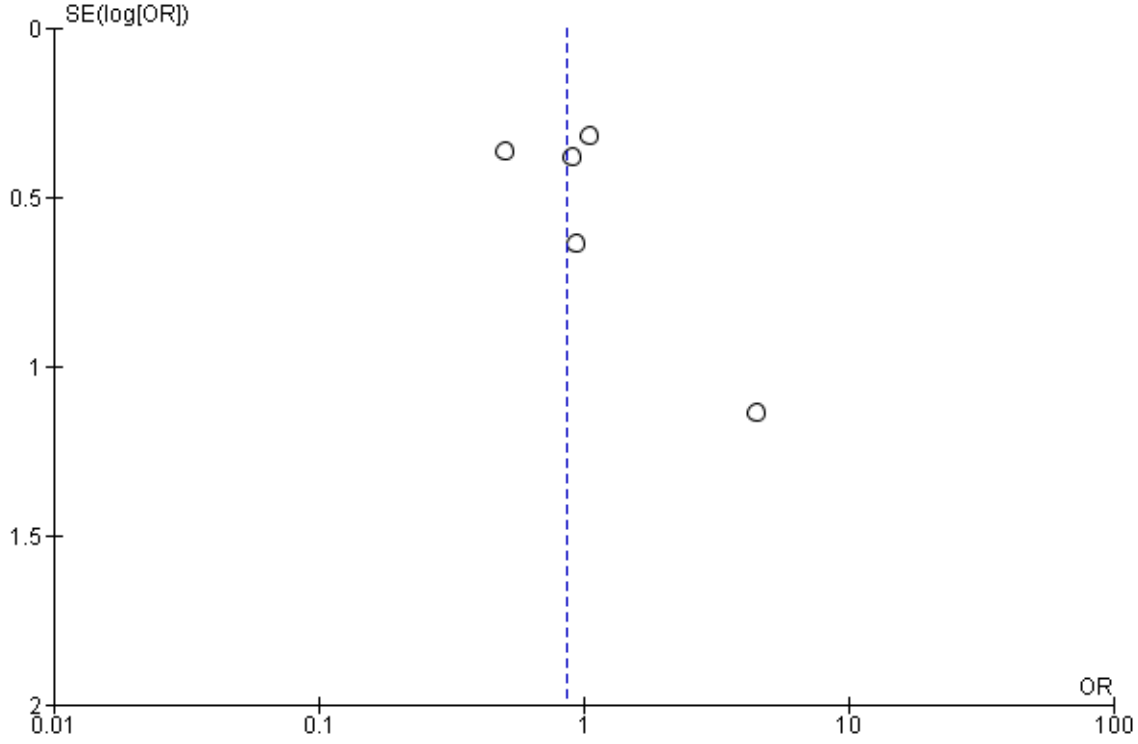
Supplementary Figure 148: Forest plot for *IL1B* TaqI RFLP (rs1143634)



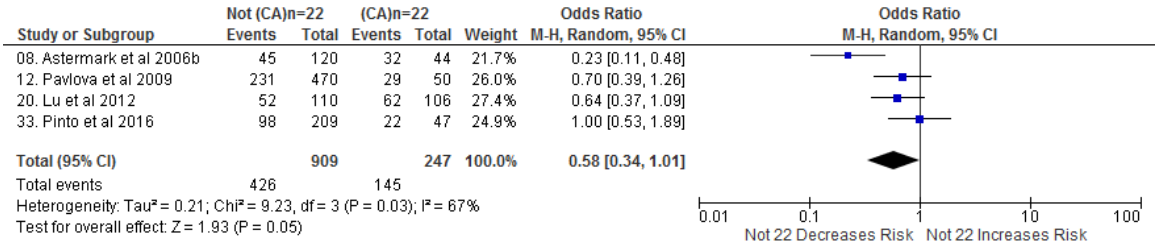
Supplementary Figure 149: Funnel plot for *IL1B* TaqI RFLP (rs1143634)



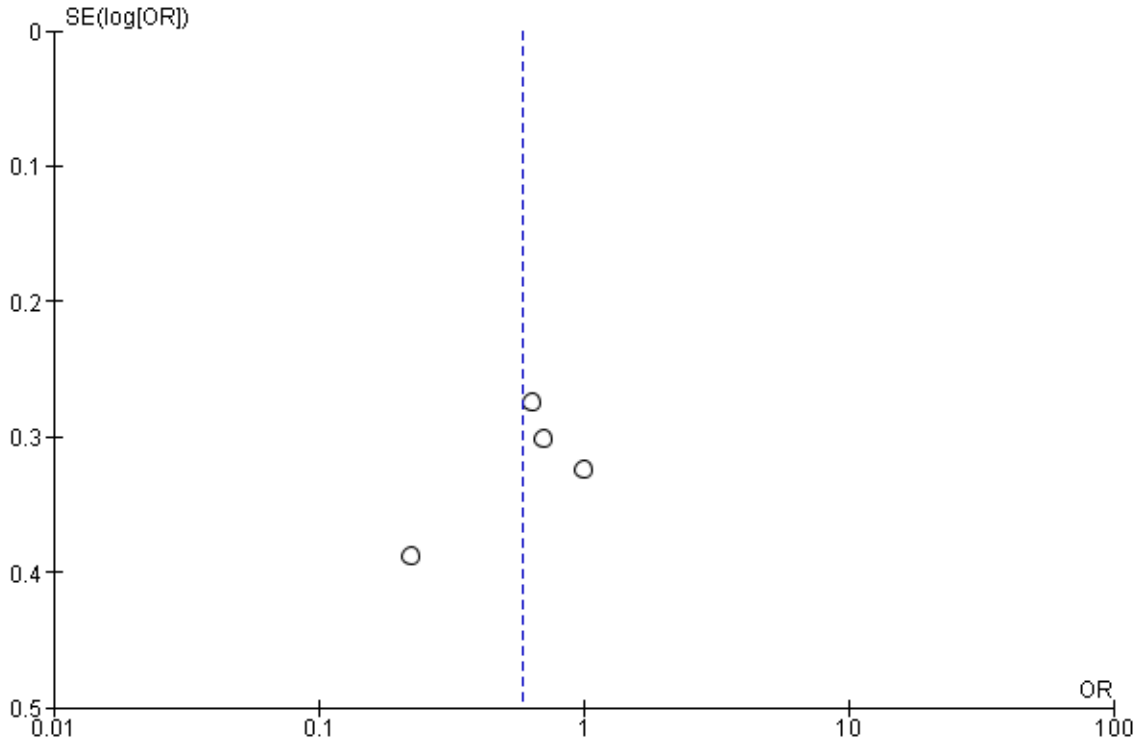
Supplementary Figure 150: Forest plot for *IL4* -590 C>T (rs2243250)



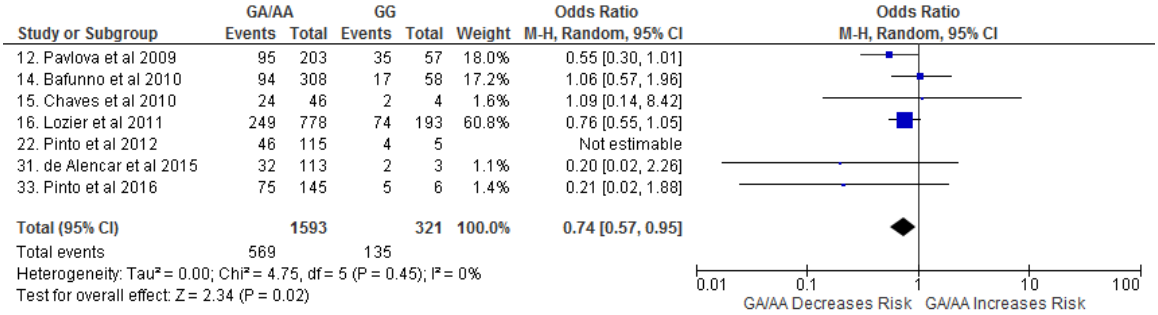
Supplementary Figure 151: Funnel plot for *IL4* -590 C>T (rs2243250)



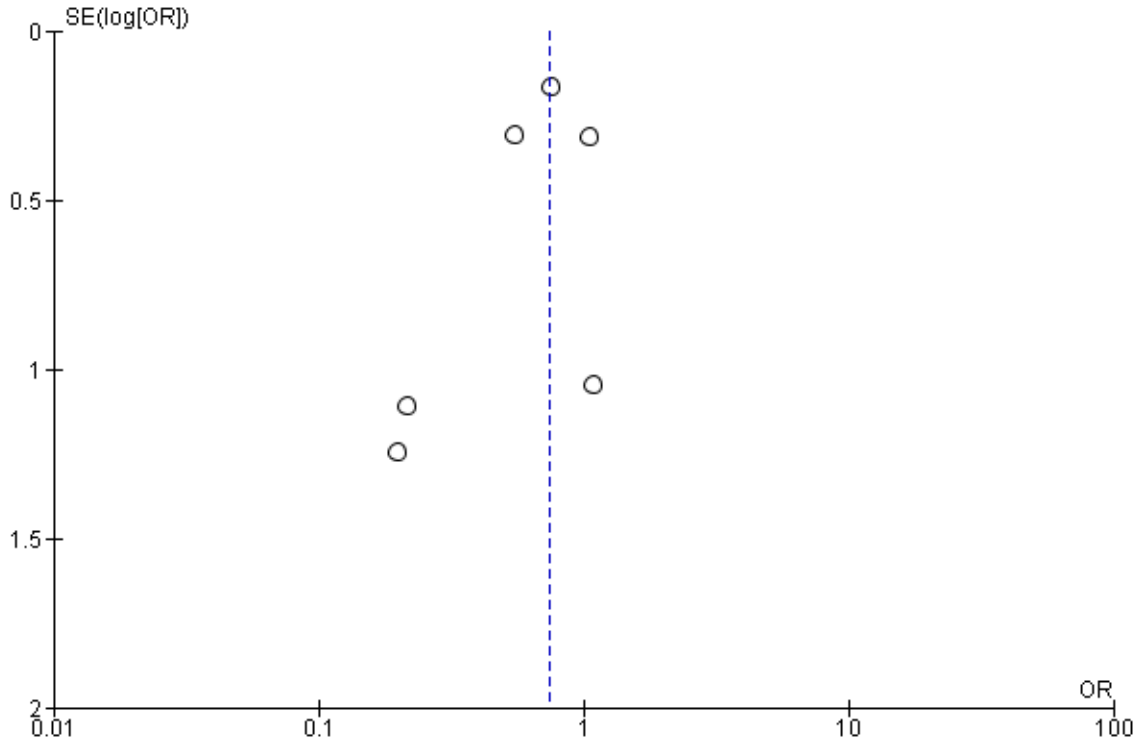
Supplementary Figure 152: Forest plot for *IL10* microsatellite (rs2234662)



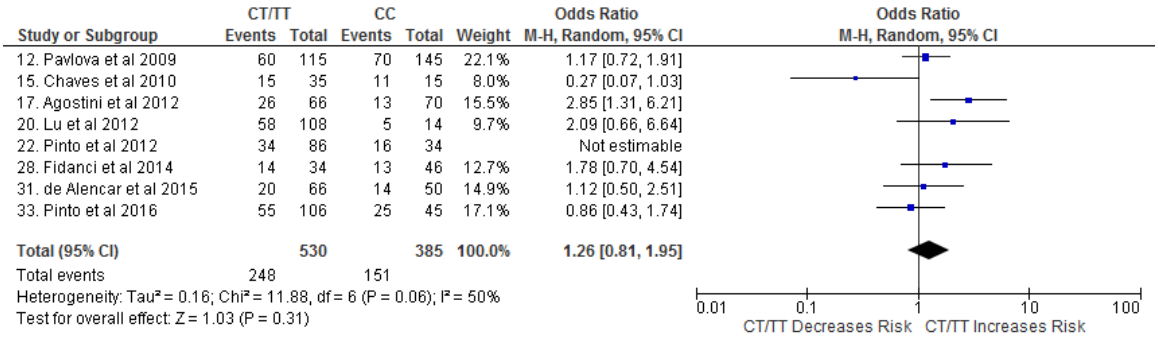
Supplementary Figure 153: Funnel plot for *IL10* microsatellite (rs2234662)



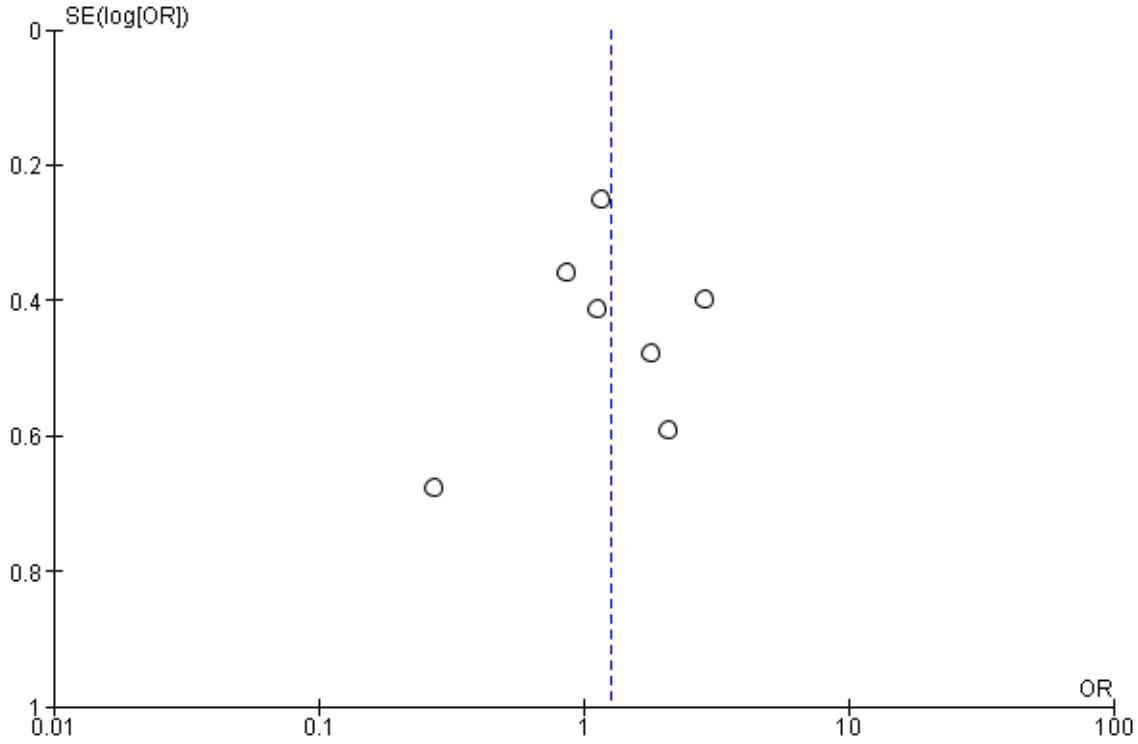
Supplementary Figure 154: Forest plot for *IL10* -1082 G>A (rs1800896)



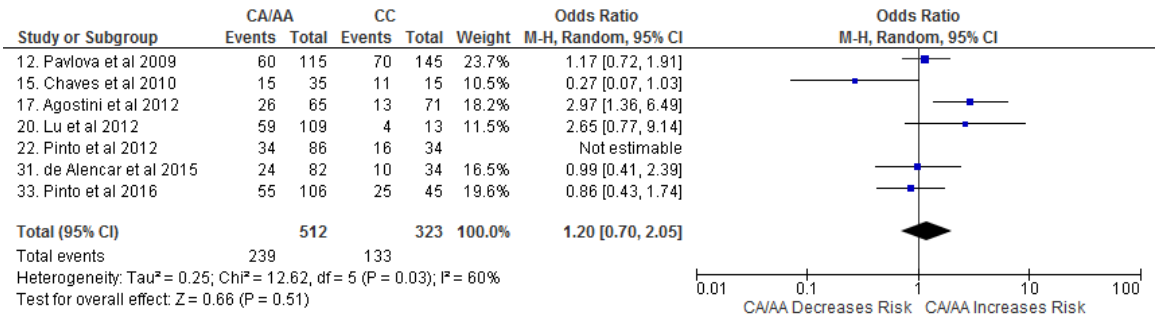
Supplementary Figure 155: Funnel plot for *IL10* -1082 G>A (rs1800896)



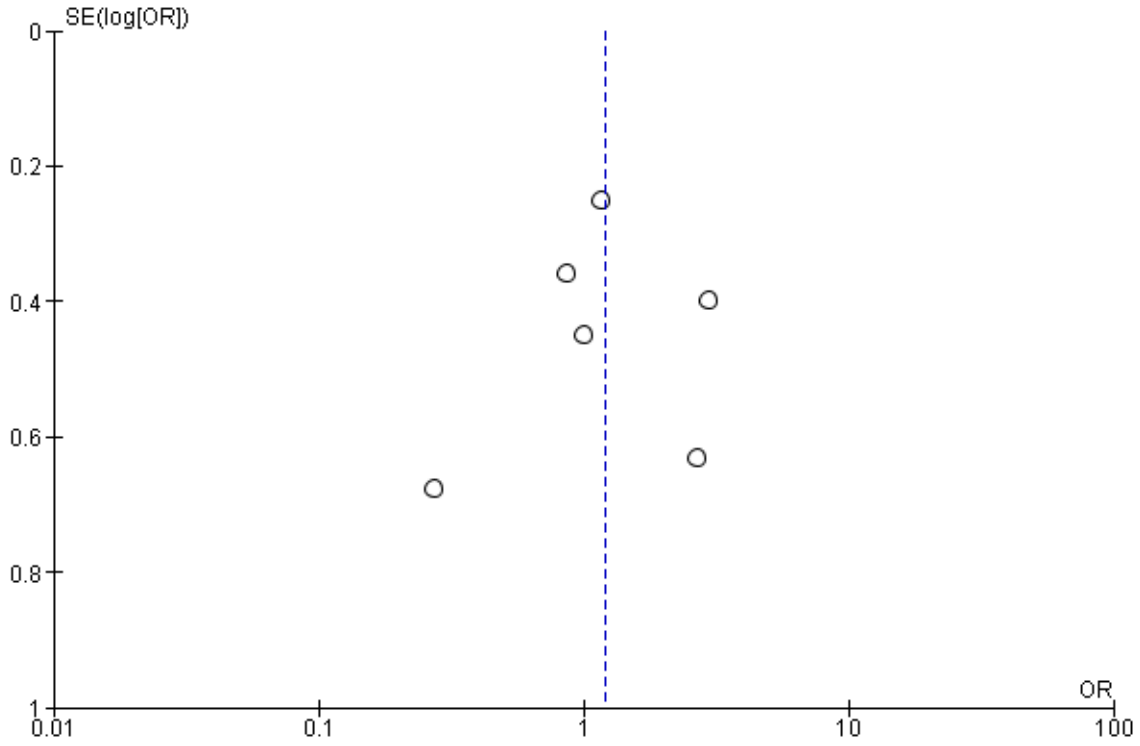
Supplementary Figure 156: Forest plot for *IL10* -819 C>T (rs1800871)



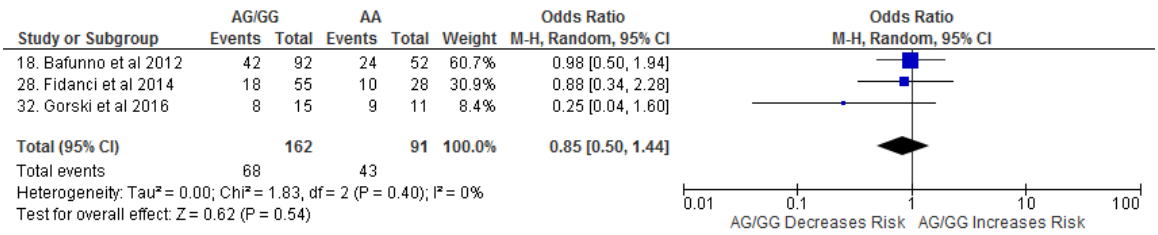
Supplementary Figure 157: Funnel plot for *IL10* -819 C>T (rs1800871)



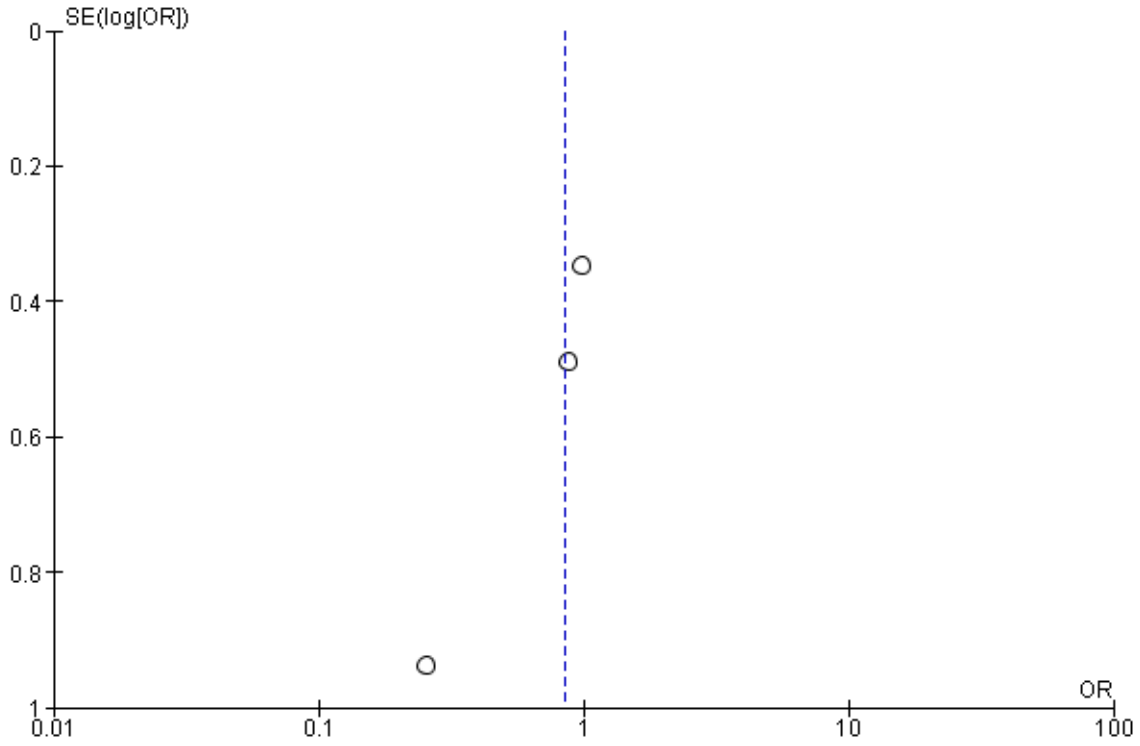
Supplementary Figure 158: Forest plot for *IL10* -592 C>A (rs1800872)



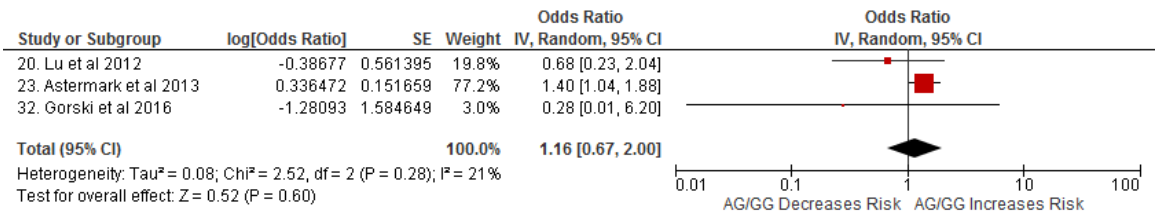
Supplementary Figure 159: Funnel plot for *IL10* -592 C>A (rs1800872)



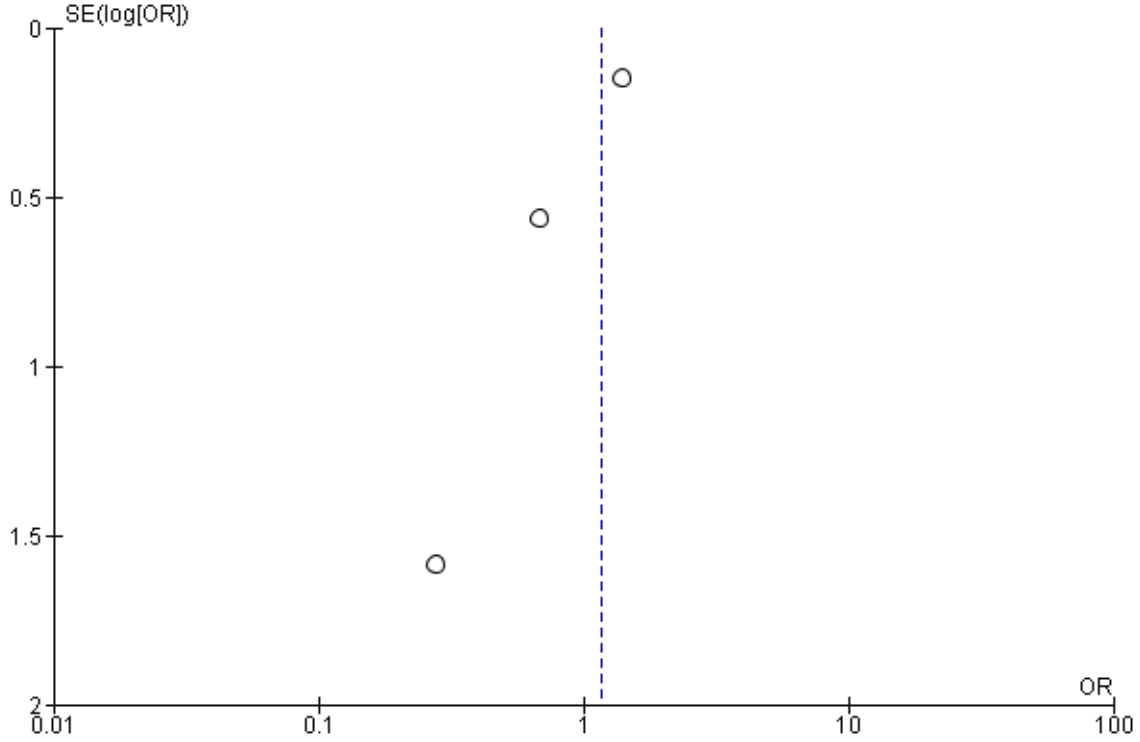
Supplementary Figure 160: Forest plot for *IL10* rs3024496



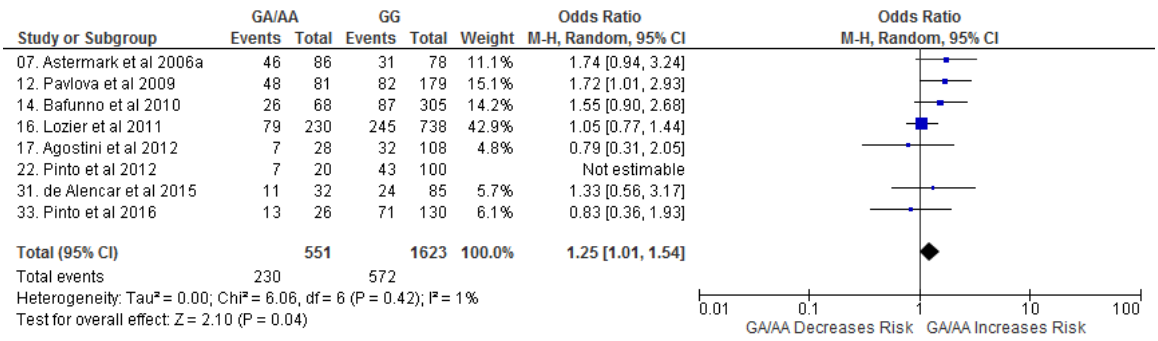
Supplementary Figure 161: Funnel plot for *IL10* rs3024496



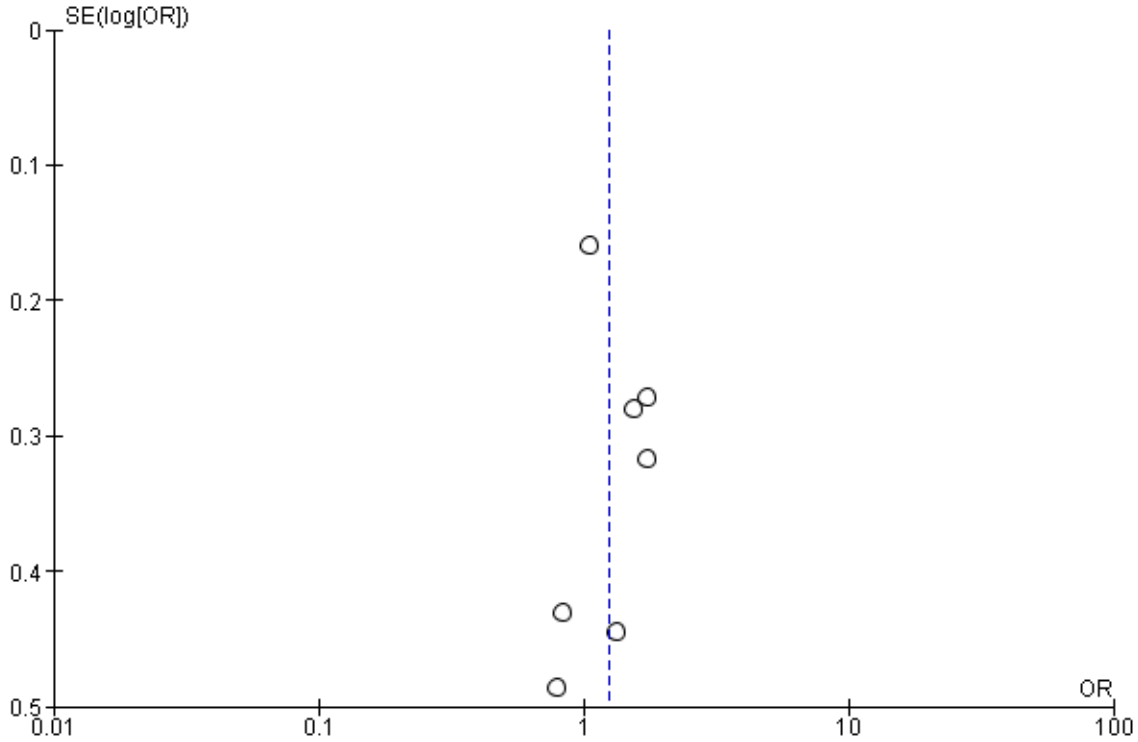
Supplementary Figure 162: Forest plot for *IL13* 2044 A>G (rs20541)



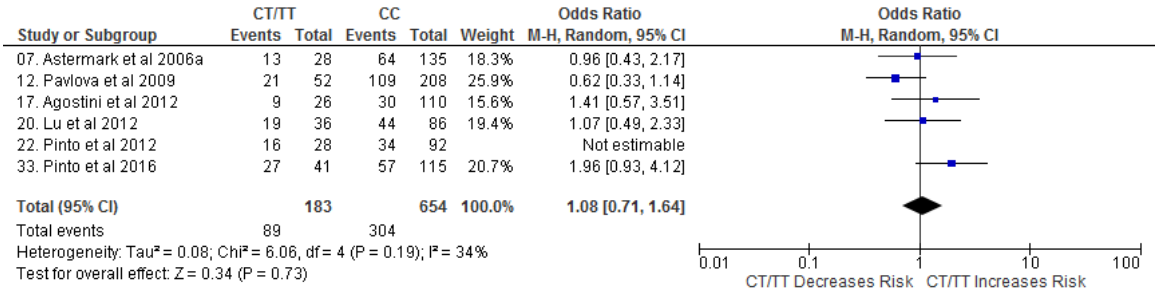
Supplementary Figure 163: Funnel plot for *IL13* 2044 A>G (rs20541)



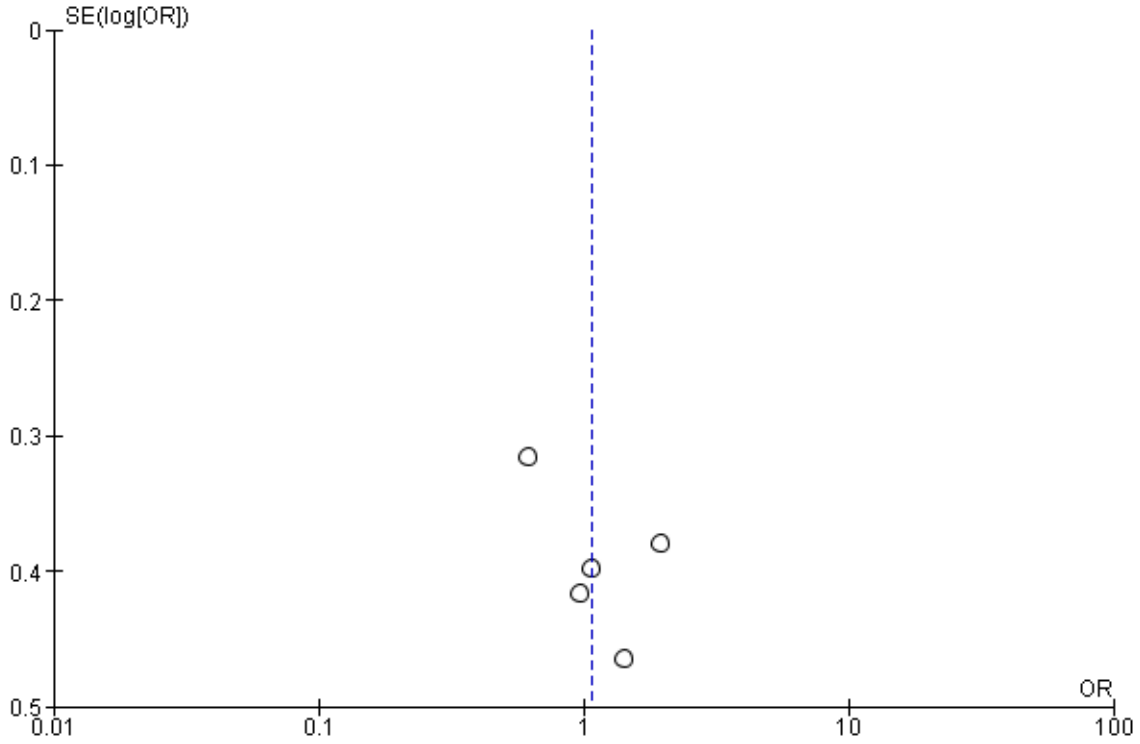
Supplementary Figure 164: Forest plot for *TNFA* -308 G>A (rs1800629)



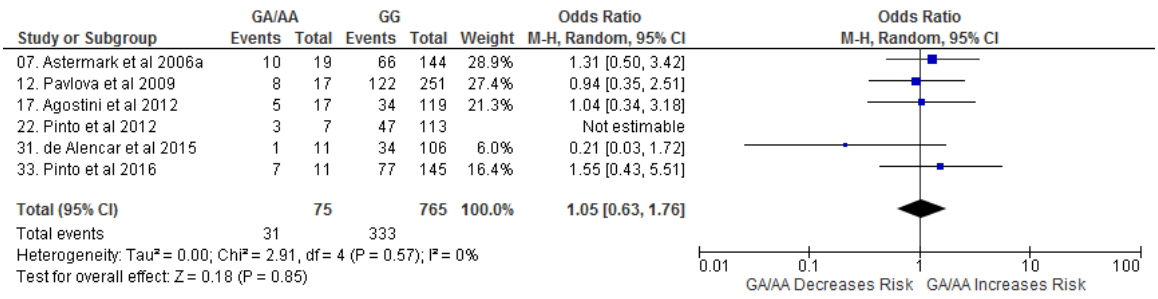
Supplementary Figure 165: Funnel plot for *TNFA* -308 G>A (rs1800629)



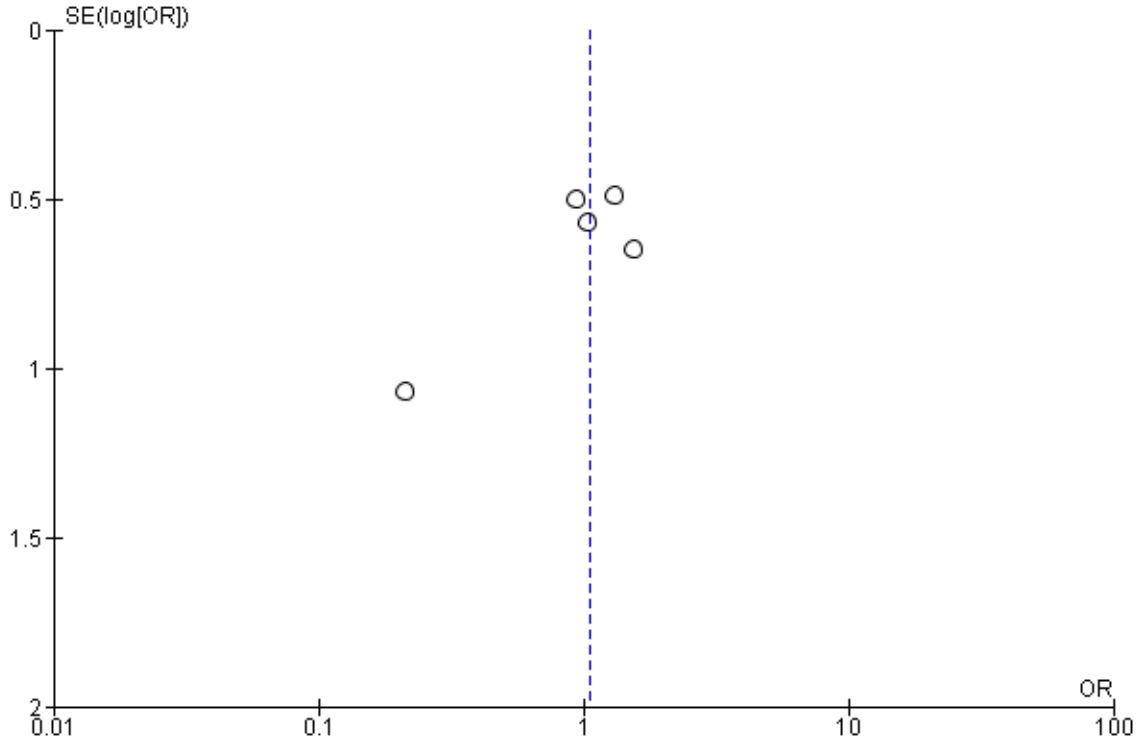
Supplementary Figure 166: Forest plot for *TNFA* -827 C>T (rs1799724)



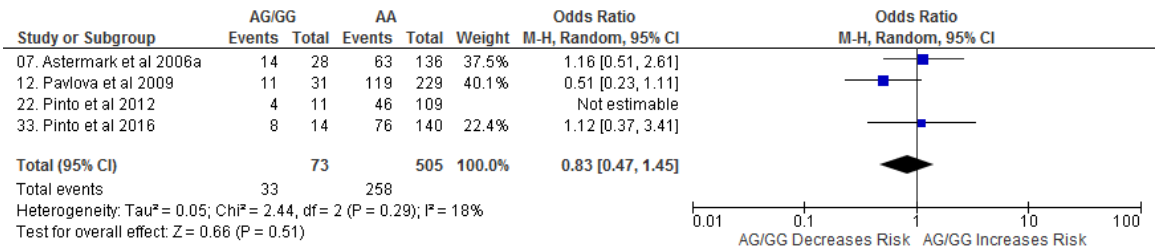
Supplementary Figure 167: Funnel plot for *TNFA* -827 C>T (rs1799724)



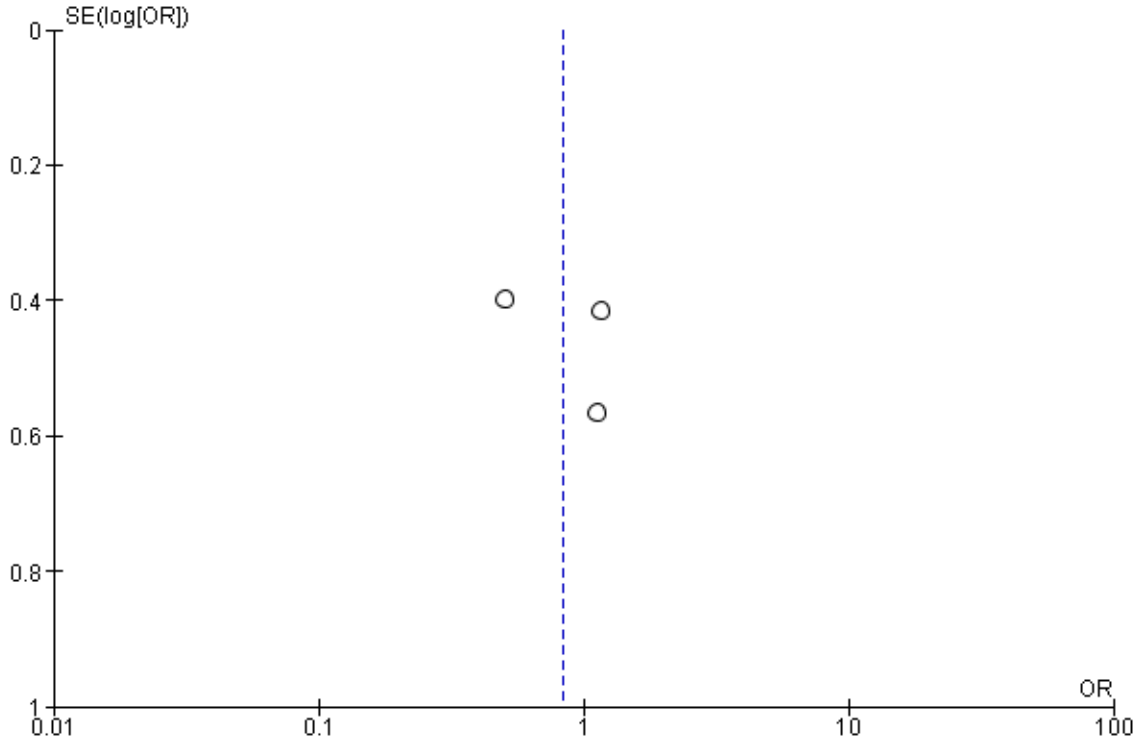
Supplementary Figure 168: Forest plot for *TNFA* -238 G>A (rs361525)



Supplementary Figure 169: Funnel plot for *TNFA* -238 G>A (rs361525)



Supplementary Figure 170: Forest plot for *TNFA* 670 A>G (rs3093662)



Supplementary Figure 171: Funnel plot for *TNFA* 670 A>G (rs3093662)

Chapter 5

	All			Mild			Moderate			Severe			Intron 22 Type 1 Inversion		
	INH (-) N=998	INH (+) N=258	OR (95% CI)	INH (-) N=224	INH (+) N=22	OR (95% CI)	INH (-) N=162	INH (+) N=24	OR (95% CI)	INH (-) N=612	INH (+) N=190	OR (95% CI)	INH (-) N=230	INH (+) N=88	OR (95% CI)
HLA-A															
***01**	153 (15.3)	32 (12.4)	REF	34 (15.2)	0 (0)	REF	24 (14.8)	6 (25.0)	REF	95 (15.5)	25 (13.2)	REF	35 (15.2)	14 (15.9)	REF
***02**	297 (29.8)	73 (28.3)	1.18 (0.73-1.9)	69 (30.8)	5 (22.7) 3 (13.6)	NE	46 (28.4)	5 (20.8)	0.43 (0.11-1.68) 0.94	182 (29.7)	57 (30.0)	1.19 (0.68-2.08) 1.55	75 (32.6)	22 (25)	0.73 (0.32-1.67)
***03**	116 (11.6)	42 (16.3)	1.59 (1.00-2.99)	28 (12.5)	2 (9.1)	NE	17 (10.5)	4 (16.7)	0.89 (0.21-4.14)	71 (11.6)	29 (15.3)	1.69 (0.81-2.97)	25 (10.9)	10 (11.4)	0.88 1.00(0.36-2.74)
***11**	63 (6.3)	21 (8.1)	1.59 (0.83-3.07)	18 (8.0)	2 (9.1)	NE	9 (5.6)	2 (8.3)	1.33 (0.14-5.74)	36 (5.9)	16 (8.4)	0.76 (0.78-3.66)	20 (8.7)	7 (8.0)	0.56 (0.29-2.67)
***23**	21 (2.1)	7 (2.7)	1.18 (0.60-4.26)	3 (1.3)	2 (9.1)	NE	3 (1.9)	1 (4.2)	0.33 (0.1-17.21)	15 (2.5)	3 (1.6)	1.08 (0.19-3.03)	9 (3.9)	2 (2.3)	0.87 (0.10-3.16)
***24**	89 (8.9)	22 (8.5)	1.26 (0.63-2.23)	17 (7.6)		NE	12 (7.4)	1 (4.2)	0.80 (0.03-3.46)	60 (9.8)	17 (9.0)	1.69 (0.52-2.24)	23 (10)	8 (9.1)	2.50 (0.30-2.53)
***25**	19 (1.9)	5 (1.9)	0.35 (0.41-3.82)	5 (2.2)	0 (0)	NE	5 (3.1)	1 (4.2)	0.20 (0.07-9.22)	9 (1.5)	4 (2.1)	0.20 (0.45-6.33)	2 (0.9)	2 (2.3)	0.31 (0.29-21.69)
***26**	27 (2.7)	2 (0.8)	0.98 (0.07-1.69)	5 (2.2)	0 (0) 1 (4.5)	NE	3 (1.9)	0 (0)	NE	19 (3.1)	1 (0.5)	0.89 (0.02-1.74)	8 (3.5)	1 (1.1)	2.50 (0.03-3.06)
***29**	44 (4.4)	9 (3.5)	1.69 (0.42-2.30)	9 (4.0)	1 (4.5)	NE	5 (3.1)	0 (0)	0.50 NE	30 (4.9)	7 (3.7)	1.60 (0.33-2.36)	4 (1.7)	4 (4.6)	3.75 (0.51-12.33)
***30**	34 (3.4)	12 (4.7)	1.29 (0.76-3.75)	7 (3.1)		NE	8 (4.9)	1 (4.2)	(0.05-5.40)	19 (3.1)	8 (4.2)	1.52 (0.6-4.28)	4 (1.7)	6 (6.8)	0.83 (0.85-16.49)
***31**	26 (2.6)	7 (2.7)	1.1 (0.49-3.38)	4 (1.8)	0 (0) 2 (9.1)	NE	7 (4.3)	0 (0)	NE 0.57	15 (2.5)	6 (3.2)	1.09 (0.51-4.56)	6 (2.6)	2 (2.3)	1.87 (0.14-5.06)
***32**	39 (3.9)	9 (3.5)	1.59 (0.47-2.61)	11 (4.9)		NE	7 (4.3)	1 (4.2)	(0.05-6.26)	21 (3.4)	6 (3.2)	1.90 (0.38-3.13)	4 (1.7)	3 (3.4)	(0.34-10.29)
***33**	6 (0.6)	2 (0.8)	(0.28-8.98)	1 (0.4)	0 (0)	NE	1 (0.6)	0 (0)	NE	4 (0.7)	2 (1.1)	(0.30-12.00)	1 (0.4)	0 (0)	NE
***34**	3 (0.3)	0 (0)	NE	0 (0)	0 (0)	NE	1 (0.6)	0 (0)	NE	2 (0.3)	0 (0)	NE	2 (0.9)	0 (0)	NE
***36**	1 (0.1)	0 (0)	NE 2.87	0 (0)	0 (0)	NE	0 (0)	0 (0)	NE 4.00	1 (0.2)	0 (0)	NE 3.80	0 (0)	0 (0)	NE
***66**	5 (0.5)	3 (1.2)	(0.60-13.61)	2 (0.9)	0 (0) 4 (18.2)	NE	1 (0.6)	1 (4.2)	(0.19-85.41) 0.31	2 (0.3)	2 (1.1)	(0.46-31.39) 0.86	0 (0)	2 (2.3)	NE 1.04
***68**	55 (5.5)	12 (4.7)	1.04 (0.48-2.25)	11 (4.9)		NE	13 (8.0)	1 (4.2)	(0.03-3.18)	31 (5.1)	7 (3.7)	(0.32-2.28)	12 (5.2)	5 (5.7)	(0.29-3.73)
HLA-B															
***07**	125 (12.5)	31 (12.0)	REF 0.76	25 (11.2)	2 (9.1)	REF	18 (11.1)	1 (4.2)	REF 2.84	82 (13.4)	25 (13.2)	REF 0.84	26 (11.3)	13 (14.8)	REF 0.71
***08**	106 (10.6)	20 (7.8)	(0.40-1.46) 2.10	21 (9.4)	0 (0)	NE 2.08	19 (11.7)	3 (12.5)	(0.24-33.71) 9.00	66 (10.8)	17 (9.0)	(0.41-1.76) 1.53	28 (12.2)	10 (11.4)	(0.25-2.01) 2.00
***13**	25 (2.5)	13 (5.0)	(0.93-4.75) 1.64	6 (2.7)	1 (4.6	(0.14-30.73) 2.50	4 (2.5)	2 (8.3)	(0.57-143.34) 18.00	15 (2.5)	7 (3.7)	(0.53-4.39) 1.09	4 (1.7)	4 (4.6)	(0.4-10.07) 0.80
***14**	32 (3.2)	13 (5.0)	(0.74-3.62) 1.48	5 (2.2)	1 (4.6)	(0.17-37.85)	3 (1.9)	3 (12.5)	(1.21-268.74) 1.13	24 (3.9)	8 (4.2)	(0.42-2.87) 1.80	10 (4.4)	4 (4.6)	(0.20-3.26) 0.67
***15**	68 (6.8)	25 (9.7)	(0.79-2.80)	10 (4.5)	0 (0)	NE	16 (9.9)	1 (4.2)	(0.06-22.55)	42 (6.9)	23 (12.1)	(0.88-3.66)	24 (10.4)	8 (9.1)	(0.22-1.99)

			1.07						6.00			1.06			0.75
***18**	49 (4.9)	13 (5.0)	(0.50-2.30)	9 (4.0)	0 (0)	NE	9 (5.6)	3 (12.5)	(0.48-74.79)	31 (5.1)	10 (5.3)	(0.44-2.56)	8 (3.5)	3 (3.4)	(0.16-3.57)
			0.44						4.50			0.31			0.11
***27**	46 (4.6)	5 (1.9)	(0.15-1.26)	10 (4.5)	0 (0)	NE	4 (2.5)	1 (4.2)	(0.20-102.72)	32 (5.2)	3 (1.6)	(0.08-1.16)	18 (7.8)	1 (1.1)	(0.01-1.03)
			1.14			2.08			3.00			1.09			0.43
***35**	81 (8.1)	23 (8.9)	(0.60-2.17)	18 (8.0)	3 (13.6)	(0.29-15.17)	12 (7.4)	2 (8.3)	(0.21-41.92)	51 (8.3)	17 (9.0)	(0.52-2.30)	23 (10)	5 (5.7)	(0.13-1.49)
			4.03						9.00			16.40			
***37**	6 (0.6)	6 (2.3)	(1.14-14.20)	3 (1.3)	0 (0)	NE	2 (1.2)	1 (4.2)	(0.33-242.33)	1 (0.2)	5 (2.6)	(1.64-164.42)	0 (0)	3 (3.4)	NE
			0.18												
***38**	22 (2.2)	1 (0.4)	(0.02-1.57)	3 (1.3)	0 (0)	NE	6 (3.7)	0 (0)	NE	13 (2.1)	0 (0)	NE	6 (2.6)	0 (0)	NE
			1.44			5.00						0.47			1.00
***39**	14 (1.4)	5 (1.9)	(0.46-4.55)	5 (2.2)	2 (9.1)	(0.50-49.57)	2 (1.2)	0 (0)	NE	7 (1.1)	1 (0.5)	(0.05-4.45)	2 (0.9)	1 (1.1)	(0.07-13.71)
			1.12			1.44			2.40			1.25			1.09
***40**	83 (8.3)	23 (8.9)	(0.59-2.11)	26 (11.6)	3 (13.6)	(0.20-10.31)	15 (9.3)	2 (8.3)	(0.17-33.09)	42 (6.9)	16 (8.4)	(0.58-2.69)	11 (4.8)	6 (6.8)	(0.31-3.84)
			1.61						6.00			1.97			
***41**	10 (1)	4 (1.6)	(0.45-5.84)	2 (0.9)	0 (0)	NE	3 (1.9)	1 (4.2)	(0.25-144.86)	5 (0.8)	3 (1.6)	(0.41-9.52)	0 (0)	2 (2.3)	NE
			0.67			1.06			0.90			0.64			0.74
***44**	175 (17.5)	29 (11.2)	(0.37-1.20)	47 (21.0)	4 (18.2)	(0.17-6.80)	20 (12.4)	1 (4.2)	(0.05-17.88)	108 (17.7)	21 (11.1)	(0.32-1.26)	35 (15.2)	13 (14.8)	(0.28-1.96)
			2.69			25.00						2.19			
***45**	6 (0.6)	4 (1.6)	(0.67-10.82)	1 (0.5)	2 (9.1)	(1.32-474)	2 (1.2)	0 (0)	NE	3 (0.5)	2 (1.1)	(0.31-15.19)	0 (0)	0 (0)	NE
***47**	2 (0.2)	0 (0)	NE	1 (0.5)	0 (0)	NE	0 (0)	0 (0)	NE	1 (0.2)	0 (0)	NE	0 (0)	0 (0)	NE
***48**	2 (0.2)	0 (0)	NE	1 (0.5)	0 (0)	NE	0 (0)	0 (0)	NE	1 (0.2)	0 (0)	NE	0 (0)	0 (0)	NE
			1.44									1.64			1.33
***49**	14 (1.4)	5 (1.9)	(0.46-4.55)	2 (0.9)	0 (0)	NE	4 (2.5)	0 (0)	NE	8 (1.3)	4 (2.1)	(0.43-6.30)	3 (1.3)	2 (2.3)	(0.18-9.92)
			1.73									0.94			0.67
***50**	7 (0.7)	3 (1.2)	(0.39-7.59)	0 (0)	0 (0)	NE	0 (0)	0 (0)	NE	7 (1.1)	2 (1.1)	(0.17-5.22)	3 (1.3)	1 (1.1)	(0.06-7.96)
			1.39			2.50			1.29			1.77			1.17
***51**	55 (5.5)	19 (7.4)	(0.70-2.77)	15 (6.7)	3 (13.6)	(0.34-18.42)	14 (8.6)	1 (4.2)	(0.06-25.94)	26 (4.3)	14 (7.4)	(0.77-4.05)	12 (5.2)	7 (8.0)	(0.35-3.89)
			0.45												
***52**	9 (0.9)	1 (0.4)	(0.05-4.09)	1 (0.5)	0 (0)	NE	1 (0.6)	0 (0)	NE	7 (1.1)	0 (0)	NE	2 (0.9)	0 (0)	NE
***53**	5 (0.5)	0 (0)	NE	3 (1.3)	0 (0)	NE	1 (0.6)	0 (0)	NE	1 (0.2)	0 (0)	NE	1 (0.4)	0 (0)	NE
			0.34									0.36			
***55**	12 (1.2)	1 (0.4)	(0.04-2.98)	2 (0.9)	0 (0)	NE	1 (0.6)	0 (0)	NE	9 (1.5)	1 (0.5)	(0.04-3.36)	4 (1.7)	0 (0)	NE
			1.01									1.09			
***56**	4 (0.4)	1 (0.4)	(0.10-10.46)	0 (0)	0 (0)	NE	1 (0.6)	0 (0)	NE	3 (0.5)	1 (0.5)	(0.10-12.35)	2 (0.9)	0 (0)	NE
			1.54			1.79			12.00			1.37			1.67
***57**	34 (3.4)	13 (5.0)	(0.70-3.39)	7 (3.1)	1 (4.6)	(0.12-25.84)	3 (1.9)	2 (8.3)	(0.71-203.60)	24 (3.9)	10 (5.3)	(0.55-3.39)	6 (2.6)	5 (5.7)	(0.40-6.97)
***58**	6 (0.6)	0 (0)	NE	1 (0.5)	0 (0)	NE	2 (1.2)	0 (0)	NE	3 (0.5)	0 (0)	NE	2 (0.9)	0 (0)	NE
HLA-C															
***01**	26 (2.6)	4 (1.6)	REF	7 (3.1)	1 (4.6)	REF	3 (1.9)	1 (4.2)	REF	16 (2.6)	2 (1.1)	REF	6 (2.6)	0 (0)	REF
			1.69			2.33						2.12			
***02**	50 (5.0)	13 (5.0)	(0.47-6.07)	9 (4.0)	3 (13.6)	(0.17-31.27)	7 (4.3)	0 (0)	NE	34 (5.6)	9 (4.7)	(0.38-11.91)	19 (8.3)	5 (5.7)	NE
			1.98			0.23			0.36			3.52			
***03**	131 (13.1)	40 (15.5)	(0.62-6.38)	31 (13.8)	1 (4.65)	(0.01-4.71)	25 (15.4)	3 (12.5)	(0.02-5.31)	75 (12.3)	33 (17.5)	(0.71-17.50)	35 (15.2)	12 (13.6)	NE
			1.73			1.08			0.38			2.67			
***04**	105 (10.5)	28 (10.9)	(0.53-5.70)	26 (11.6)	4 (18.2)	(0.09-12.66)	16 (9.9)	2 (8.3)	(0.02-6.40)	63 (10.3)	21 (11.1)	(0.52-13.61)	28 (12.2)	9 (10.2)	NE
			1.39			0.44			0.75			1.90			
***05**	103 (10.3)	22 (8.5)	(0.42-4.64)	32 (14.3)	2 (9.1)	(0.03-6.29)	12 (7.4)	3 (12.5)	(0.05-11.44)	59 (9.7)	14 (7.4)	(0.36-10.00)	18 (7.8)	8 (9.1)	NE
			2.93			1.65			1.25			3.62			
***06**	82 (8.2)	37 (14.34)	(0.90-9.54)	17 (7.6)	4 (18.2)	(0.14-19.71)	12 (7.4)	5 (20.8)	(0.09-17.16)	53 (8.7)	24 (12.6)	(0.71-18.41)	15 (6.5)	11 (12.5)	NE

***07**	307 (30.8)	67 (26.0)	1.42 (0.45-4.44)	61 (27.2)	4 (18.2)	0.46 (0.04-5.29)	53 (32.7)	5 (20.8)	0.28 (0.02-3.69)	193 (31.5)	53 (27.9)	2.20 (0.45-10.64)	72 (31.3)	28 (31.8)	NE
***08**	33 (3.3)	12 (4.7)	2.36 (0.64-8.73)	6 (2.7)	1 (4.6)	1.17 (0.05-26.70)	3 (1.9)	3 (12.5)	3.00 (0.16-55.25)	24 (3.9)	7 (3.7)	2.33 (0.39-13.84)	9 (3.9)	3 (3.4)	NE
***12**	57 (5.7)	12 (4.7)	1.37 (0.38-4.95)	8 (3.6)	2 (9.1)	1.75 (0.11-27.07)	11 (6.8)	0 (0)	NE	38 (6.2)	7 (3.7)	1.47 (0.25-8.58)	14 (6.1)	2 (2.3)	NE
***14**	16 (1.6)	2 (0.8)	0.81 (0.12-5.43)	5 (2.2)	0 (0)	NE	2 (1.2)	0 (0)	NE	9 (1.5)	2 (1.1)	1.78 (0.19-16.56)	3 (1.3)	0 (0)	NE
***15**	27 (2.7)	6 (2.3)	1.44 (0.34-6.13)	6 (2.7)	0 (0)	NE	8 (4.9)	0 (0)	NE	13 (2.1)	6 (3.2)	3.69 (0.58-23.47)	5 (2.2)	3 (3.4)	NE
***16**	51 (5.1)	11 (4.3)	1.40 (0.38-5.15)	13 (5.8)	0 (0)	NE	8 (4.9)	1 (4.2)	0.38 (0.01-9.48)	30 (4.9)	9 (4.7)	2.40 (0.42-13.56)	6 (2.6)	5 (5.7)	NE
***17**	10 (1.0)	4 (1.6)	2.60 (0.5-13.48)	3 (1.3)	0 (0)	NE	2 (1.2)	1 (4.2)	1.50 (0.05-48.09)	5 (0.8)	3 (1.6)	4.80 (0.56-41.47)	0 (0)	2 (2.3)	NE

Supplementary Table 1: Association between Major Histocompatibility Complex Class I alleles and inhibitors, overall, by severity, and among persons with intron 22 Type 1 inversion among enrollees in the Hemophilia Inhibitor Research Study

	All			Mild			Moderate			Severe			Intron 22 Type 1 Inversion		
	INH (-) N=998	INH (+) N=258	OR (95% CI)	INH (-) N=224	INH (+) N=22	OR (95% CI)	INH (-) N=162	INH (+) N=24	OR (95% CI)	INH (-) N=612	INH (+) N=190	OR (95% CI)	INH (-) N=230	INH (+) N=88	OR (95% CI)
HLA-DPB1															
***01**	57 (5.7)	6 (2.3)	REF	16 (7.1)	1 (4.6)	REF	8 (4.9)	0 (0)	REF	33 (5.4)	5 (2.6)	REF	18 (7.8)	2 (2.3)	REF
***02**	143 (14.3)	38 (14.7)	2.52 (0.97-6.60)	28 (12.5)	2 (9.1)	1.14 (0.08-15.45)	29 (17.9)	4 (16.7)	NE	86 (14.1)	30 (15.8)	2.30 (0.78-6.79)	23 (10.0)	13 (14.8)	5.09 (0.94-27.67)
***03**	116 (11.6)	32 (12.4)	2.62 (0.99-6.95)	27 (12.1)	2 (9.1)	1.19 (0.09-16.04)	20 (12.4)	5 (20.8)	NE	69 (11.3)	23 (12.1)	2.20 (0.73-6.65)	23 (10.0)	8 (9.1)	3.13 (0.54-18.07)
***04**	532 (53.3)	141 (54.7)	2.52 (1.02-6.23)	121 (54.0)	12 (54.6)	1.59 (0.17-14.51)	85 (52.5)	11 (45.8)	NE	326 (53.3)	104 (54.7)	2.11 (0.76-5.81)	134 (58.3)	47 (53.4)	3.16 (0.65-15.24)
***05**	31 (3.1)	9 (3.5)	2.76 (0.85-8.97)	7 (3.1)	0 (0)	NE	3 (1.9)	1 (4.2)	NE	21 (3.4)	6 (3.2)	1.89 (0.48-7.45)	8 (3.5)	1 (1.1)	1.13 (0.08-16.25)
***09**	13 (1.3)	4 (1.6)	2.92 (0.67-12.75)	1 (0.6)	0 (0)	NE	0 (0)	1 (4.2)	NE	12 (2.0)	3 (1.6)	1.65 (0.31-8.65)	3 (1.3)	1 (1.1)	3.00 (0.18-50.90)
***10**	23 (2.3)	6 (2.3)	2.48 (0.68-9.04)	8 (3.6)	1 (4.6)	2.00 (0.10-42.09)	4 (2.5)	1 (4.2)	NE	11 (1.8)	4 (2.1)	2.40 (0.51-11.39)	3 (1.3)	4 (4.6)	12.00 (1.33-108.13)
***11**	29 (2.9)	4 (1.6)	1.31 (0.32-5.37)	8 (3.6)	0 (0)	NE	3 (1.9)	0 (0)	NE	18 (2.9)	3 (1.6)	1.10 (0.22-5.56)	3 (1.3)	2 (2.3)	6.00 (0.53-68.00)
***13**	16 (1.6)	7 (2.7)	4.16 (1.15-15.04)	3 (1.3)	2 (9.1)	10.67 (0.63-181.91)	4 (2.5)	0 (0)	NE	9 (1.5)	4 (2.1)	2.93 (0.60-14.30)	4 (1.7)	5 (5.7)	11.25 (1.43-88.78)
***14**	10 (1.0)	2 (0.9)	1.90 (0.31-11.78)	1 (0.6)	0 (0)	NE	1 (0.6)	0 (0)	NE	8 (1.3)	2 (1.1)	1.65 (0.25-11.09)	5 (2.2)	1 (1.1)	1.80 (0.12-27.59)
***15**	6 (0.6)	1 (0.4)	1.58 (0.14-17.35)	2 (0.9)	0 (0)	NE	2 (1.2)	1 (4.2)	NE	2 (0.3)	0 (0)	NE	0 (0)	0 (0)	NE
***16**	8 (0.8)	0 (0)	NE	0 (0)	0 (0)	NE	2 (1.2)	0 (0)	NE	6 (1.0)	0 (0)	NE	3 (1.3)	0 (0)	NE
***17**	9 (0.9)	5 (1.9)	5.28 (1.24-22.5)	2 (0.9)	1 (4.6)	8.00 (0.30-216.38)	1 (0.6)	0 (0)	NE	6 (1.0)	4 (2.1)	4.40 (0.84-23.07)	1 (0.4)	2 (2.3)	18.00 (0.94-345.10)
***19**	5 (0.5)	3 (1.2)	5.70 (1.00-32.64)	0 (0)	1 (4.6)	NE	0 (0)	0 (0)	NE	5 (0.8)	2 (1.1)	2.64 (0.36-19.25)	2 (0.9)	2 (2.3)	9.00 (0.69-117.50)
HLA-DQA1															
***01**	412 (41.3)	111 (43.0)	REF	85 (38.0)	7 (31.8)	REF	71 (43.8)	8 (33.3)	REF	256 (41.8)	81 (42.6)	REF	100 (43.5)	36 (40.9)	REF
***02**	137 (13.7)	43 (16.7)	1.16 (0.76-1.78)	31 (13.8)	4 (18.2)	1.57 (0.40-6.11)	20 (12.4)	3 (12.5)	1.33 (0.30-5.90)	86 (14.1)	31 (16.3)	1.14 (0.69-1.89)	23 (10.0)	17 (19.3)	2.05 (0.95-4.44)
***03**	173 (17.3)	36 (14.0)	0.77 (0.50-1.20)	47 (21.0)	2 (9.1)	0.52 (0.10-2.81)	24 (14.8)	2 (8.3)	0.74 (0.14-4.05)	102 (16.7)	31 (16.3)	0.96 (0.58-1.58)	48 (20.9)	15 (17.1)	0.87 (0.42-1.80)
***04**	21 (2.1)	5 (1.9)	0.88 (0.31-2.52)	5 (2.2)	0 (0)	NE	4 (2.5)	1 (4.2)	2.22 (0.20-25.15)	12 (2.0)	4 (2.1)	1.05 (0.31-3.56)	7 (3.0)	3 (3.4)	1.19 (0.27-5.21)
***05**	251 (25.2)	62 (24.0)	0.92 (0.64-1.32)	55 (24.6)	9 (40.9)	1.99 (0.66-5.95)	43 (26.5)	10 (41.7)	2.06 (0.72-5.93)	153 (25.0)	42 (22.1)	0.87 (0.56-1.35)	51 (22.2)	17 (19.3)	0.93 (0.46-1.87)
***06**	4 (0.4)	1 (0.4)	0.93 (0.09-9.38)	1 (0.6)	0 (0)	NE	0 (0)	0 (0)	NE	3 (0.5)	1 (0.5)	1.05 (0.10-11.53)	1 (0.4)	0 (0)	NE
HLA-DQB1															
***02**	228 (22.9)	52 (20.2)	0.88 (0.54-1.42)	53 (23.7)	3 (13.6)	0.58 (0.10-3.35)	38 (23.5)	7 (29.2)	1.60 (0.35-7.26)	137 (22.4)	37 (19.5)	0.87 (0.49-1.55)	45 (19.6)	17 (19.3)	1.16 (0.49-2.75)
***03**	343 (34.4)	90 (34.9)	1.01 (0.65-1.57)	81 (36.2)	12 (54.6)	1.53 (0.38-6.20)	52 (32.1)	8 (33.3)	1.33 (0.30-5.86)	210 (34.3)	68 (35.8)	1.05 (0.62-1.76)	80 (34.8)	32 (36.4)	1.23 (0.57-2.64)
***04**	21 (2.1)	4 (1.6)	0.73 (0.23-2.39)	5 (2.2)	0 (0)	NE	4 (2.5)	1 (4.2)	2.17 (0.16-29.86)	12 (2.0)	3 (1.6)	0.81 (0.20-3.27)	7 (3.0)	2 (2.3)	0.88 (0.15-5.15)
***05**	154 (15.4)	40 (15.5)	REF	31 (13.8)	3 (13.6)	REF	26 (16.1)	3 (12.5)	REF	97 (15.9)	30 (15.8)	REF	43 (18.7)	14 (15.9)	REF

			1.10			0.77			1.03			1.08			1.28
***06**	252 (25.3)	72 (27.9)	(0.70-1.74)	54 (24.1)	4 (18.2)	(0.15-3.95)	42 (25.9)	5 (20.8)	(0.21-5.06)	156 (25.5)	52 (27.4)	(0.63-1.85)	55 (23.9)	23 (26.1)	(0.57-2.90)
HLA-DRB1															
					2 (9.1)										
***01**	108 (10.8)	25 (9.7)	REF	23 (10.3)		REF	21 (13.0)	2 (8.3)	REF	64 (10.5)	19 (10.0)	REF	32 (13.9)	8 (9.1)	REF
			0.82						1.83			0.91			0.80
***03**	132 (13.2)	25 (9.7)	(0.43-1.55)	31 (13.8)	0 (0)	NE	23 (14.2)	4 (16.7)	(0.28-12.08)	78 (12.8)	21 (11.1)	(0.43-1.90)	30 (13.0)	6 (6.8)	(0.23-2.74)
			0.95		2 (9.1)	0.53			0.95			1.07			1.36
***04**	159 (15.9)	35 (13.6)	(0.52-1.73)	43 (19.2)		(0.06-4.49)	22 (13.6)	2 (8.3)	(0.11-8.23)	94 (15.4)	30 (15.8)	(0.54-2.14)	44 (19.1)	15 (17.1)	(0.49-3.79)
			1.33			1.59		3 (12.5)	1.58			1.16			2.56
***07**	136 (13.6)	42 (16.3)	(0.74-2.39)	29 (13.0)	4 (18.2)	(0.24-10.34)	20 (12.4)		(0.22-11.50)	87 (14.2)	30 (15.8)	(0.58-2.32)	25 (10.9)	16 (18.2)	(0.90-7.30)
			0.80						1.75			0.90			1.33
***08**	27 (2.7)	5 (1.9)	(0.27-2.41)	6 (2.7)	0 (0)	NE	6 (3.7)	1 (4.2)	(0.12-25.97)	15 (2.5)	4 (2.1)	(0.25-3.22)	9 (3.9)	3 (3.4)	(0.27-6.58)
			0.27									0.42			
***09**	16 (1.6)	1 (0.4)	(0.03-2.37)	4 (1.8)	0 (0)	NE	4 (2.5)	0 (0)	NE	8 (1.3)	1 (0.5)	(0.04-4.00)	3 (1.3)	0 (0)	NE
			2.16									2.53			12.00
***10**	6 (0.6)	3 (1.2)	(0.47-9.94)	2 (0.9)	0 (0)	NE	0 (0)	0 (0)	NE	4 (0.7)	3 (1.6)	(0.48-13.32)	1 (0.4)	3 (3.4)	(0.97-148.28)
			1.28			5.11			3.50			0.77			1.60
***11**	91 (9.1)	27 (10.5)	(0.67-2.44)	18 (8.0)	8 (36.4)	(0.89-29.50)	12 (7.4)	4 (16.7)	(0.51-24.20)	61 (10.0)	14 (7.4)	(0.34-1.74)	15 (6.5)	6 (6.8)	(0.44-5.79)
			1.15									1.50			3.00
***12**	15 (1.5)	4 (1.6)	(0.33-4.01)	4 (1.8)	0 (0)	NE	2 (1.2)	0 (0)	NE	9 (1.5)	4 (2.1)	(0.39-5.77)	4 (1.7)	3 (3.4)	(0.51-17.64)
			1.50			1.70			1.91			1.37			1.63
***13**	118 (11.8)	41 (15.9)	(0.83-2.71)	27 (12.1)	4 (18.2)	(0.26-11.13)	22 (13.6)	4 (16.7)	(0.29-12.66)	69 (11.3)	28 (14.7)	(0.67-2.78)	27 (11.7)	11 (12.5)	(0.54-4.89)
			1.58			2.30			1.75			1.60			1.60
***14**	30 (3.0)	11 (4.3)	(0.67-3.74)	5 (2.2)	1 (4.6)	(0.15-34.92)	6 (3.7)	1 (4.2)	(0.12-25.97)	19 (3.1)	9 (4.7)	(0.59-4.30)	5 (2.2)	2 (2.3)	(0.24-10.76)
			1.09			0.38		3 (12.5)	1.37			0.98			1.81
***15**	146 (14.6)	37 (14.3)	(0.60-1.98)	30 (13.4)	1 (4.6)	(0.03-5.09)	23 (14.2)		(0.19-9.93)	93 (15.2)	27 (14.2)	(0.48-1.97)	31 (13.5)	14 (15.9)	(0.63-5.16)
			0.62												1.00
***16**	14 (1.4)	2 (0.8)	(0.12-3.13)	2 (0.9)	0 (0)	NE	1 (0.6)	0 (0)	NE	11 (1.8)	0 (0)	NE	4 (1.7)	1 (1.1)	(0.09-11.51)

Supplementary Table 2: Association between Major Histocompatibility Complex Class II alleles and inhibitors, overall, by severity, and among persons with intron 22 Type 1 inversion among enrollees in the Hemophilia Inhibitor Research Study

		All			Mild			Moderate			Severe			Intron 22 Type 1 Inversion		
		INH (-) N=508	**INH (+) N=134**	**OR (95% CI)**	**INH (-) N=115**	**INH (+) N=11**	**OR (95% CI)**	**INH (-) N=83**	**INH (+) N=13**	**OR (95% CI)**	**INH (-) N=310**	**INH (+) N=98**	**OR (95% CI)**	**INH (-) N=115**	**INH (+) N=46**	**OR (95% CI)**
IL1A rs17561																
	CC	233 (46.0)	79 (59.4)	REF	45 (39.1)	6 (54.6)	REF	41 (49.4)	7 (53.9)	REF	147 (47.6)	59 (60.8)	REF	54 (47.0)	29 (64.4)	REF

AC	228 (45.0)	46 (34.6)	0.58 (0.30-1.14)	58 (50.4)	5 (45.5)	0.54 (0.06-4.63)	35 (42.2)	3 (23.1)	0.84 (0.11-6.38)	135 (43.7)	33 (34.0)	0.58 (0.26-1.31)	52 (45.2)	14 (31.1)	0.49 (0.14-1.68)
AA	46 (9.1)	8 (6.0)		12 (10.4)	0 (0)		7 (8.4)	3 (23.1)		27 (8.7)	5 (5.2)		9 (7.8)	2 (4.4)	
IL12B rs1003199															
GG	121 (23.8)	46 (34.3)	REF	28 (24.4)	3 (27.3)	REF	17 (20.5)	4 (30.8)	REF	76 (24.5)	35 (35.7)	REF	29 (25.2)	17 (37.0)	REF
GA	256 (50.49)	58 (43.3)	0.60 (0.29-1.22)	57 (49.6)	7 (63.6)	0.86 (0.08-9.60)	45 (54.2)	5 (38.5)	0.58 (0.06-5.44)	154 (49.7)	41 (41.8)	0.58 (0.25-1.36)	54 (47.0)	19 (41.3)	0.58 (0.16-2.04)
AA	131 (25.8)	30 (22.4)		30 (26.1)	1 (9.19)		21 (25.3)	4 (30.8)		80 (25.8)	22 (22.5)		32 (27.8)	10 (21.7)	
CD80 rs16829984															
GG	397 (79.4)	89 (69.0)	REF	93 (82.3)	10 (90.9)	REF	61 (75.3)	9 (69.2)	REF	243 (79.4)	65 (67.7)	REF	98 (86.0)	32 (72.7)	REF
CG	95 (19.0)	37 (28.7)	1.73 0.82-3.66)	17 (15.0)	1 (9.1)	0.47 (0.01-18.05)	20 (24.7)	4 (30.8)	1.36 (0.15-12.49)	58 (19.0)	28 (29.2)	1.84 (0.76-4.45)	15 (13.2)	10 (22.7)	2.30 (0.53-9.99)
CC	8 (1.6)	3 (2.3)		3 (2.7)	0 (0)		0 (0)	0 (0)		5 (1.6)	3 (3.1)		1 (0.9)	2 (4.6)	

Supplementary Table 3: Association between variants in immune response genes outside the Major Histocompatibility Complex region and inhibitors, overall, by severity, and among persons with intron 22 Type 1 inversion among enrollees in the Hemophilia Inhibitor Research Study

CHR	SNP	LOC	GENE	Allele 1	Allele 2	INH (+)	INH (-)	P Value	No. Permutations
1	rs3024509	206943297	IL10	G	A	19/237	64/964	0.6667	14
1	rs3024493	206943968	IL10	A	C	33/211	168/824	0.2647	67
1	rs3021094	206944952	IL10	C	A	21/235	91/937	0.7778	8
1	rs1800871	206946634	IL10	A	G	59/193	219/799	0.7273	10
1	rs4072226	206957449	IL10	A	G	114/142	443/585	0.8571	6
1	rs4072227	206957558	IL10	G	A	12/244	73/955	0.2436	77
2	rs17561	113537223	IL1A	A	C	57/197	325/701	0.005453	4400
2	rs2071374	113537352	IL1A	C	A	79/173	269/749	0.8571	6
2	rs2853550	113587121	IL1B	A	G	16/240	77/951	0.4286	34
2	rs3136558	113591275	IL1B	G	A	54/202	267/761	0.0824	266
2	rs1143623	113595829	IL1B	G	C	70/186	282/746	1	6
2	rs3087263	113885768	IL1RN	A	G	30/226	100/928	0.8571	6
2	rs3181052	113886049	IL1RN	A	G	33/223	144/884	0.7273	10
2	rs380092	113888900	IL1RN	A	T	82/174	345/683	0.8571	6
2	rs397211	113892141	IL1RN	G	A	74/180	303/725	0.8571	6
2	rs3181100	204572006	CD28	C	G	106/148	419/607	0.8571	6
2	rs10490573	204583163	CD28	A	G	45/211	187/839	0.7778	8
2	rs3769684	204584759	CD28	G	A	14/242	54/974	1	6
2	rs3181107	204593726	CD28	G	A	26/230	69/959	0.06117	375
2	rs16840252	204731519	CTLA4	A	G	49/207	179/849	0.625	15
2	rs11571317	204732008	CTLA4	A	G	19/231	81/941	0.7273	10
2	rs231775	204732714	CTLA4	G	A	94/146	377/621	0.8571	6
2	rs3087243	204738919	CTLA4	A	G	102/150	451/567	0.3478	45
3	rs17281703	119243549	CD80	A	G	33/223	118/910	1	6
3	rs1599795	119243855	CD80	A	T	40/216	189/839	0.4412	33
3	rs7628626	119244421	CD80	A	C	42/214	176/850	0.7778	8
3	rs13071247	119266793	CD80	C	A	39/217	174/854	0.6923	12
3	rs6807532	119274841	CD80	A	G	17/239	74/954	1	6
3	rs1485332	119274921	CD80	G	C	64/190	211/817	0.2174	91
3	rs6810204	119275098	CD80	A	G	24/232	91/937	1	6

3	rs1385521	119275339	CD80	A	G	13/239	54/966	1	6
3	rs16829984	119278540	CD80	C	G	42/204	112/900	0.01795	1336
3	rs1880661	119278848	CD80	A	G	119/133	420/588	0.06354	361
3	rs2681404	121778970	CD86	G	A	34/222	143/885	0.8571	6
3	rs13095010	121784531	CD86	G	A	14/242	67/961	0.6923	12
3	rs4308217	121793187	CD86	A	C	75/181	353/675	0.1533	136
3	rs9282641	121796768	CD86	A	G	13/243	84/944	0.1078	203
3	rs2681417	121825197	CD86	G	A	22/234	69/959	0.3902	40
3	rs2681420	121834057	CD86	G	A	55/195	174/842	0.06389	359
3	rs17281995	121839641	CD86	G	C	36/220	160/866	1	6
3	rs2243115	159706280	IL12A	C	A	29/227	123/903	0.8571	6
3	rs583911	159710390	IL12A	G	A	112/144	429/599	0.5217	22
4	rs2069778	123376135	IL2	A	G	42/214	161/867	0.7778	8
4	rs2069777	123376437	IL2	A	G	18/238	71/957	1	6
4	rs4833248	123380405	IL2	A	G	67/189	267/751	0.6111	17
5	rs2069812	131879916	IL5	A	G	69/183	310/718	0.2639	71
5	rs2243263	132013299	IL4	G	C	31/225	106/918	0.4375	31
5	rs2243266	132013789	IL4	A	G	34/222	150/876	0.5217	22
5	rs919766	158747564	IL12B	C	A	33/223	94/928	0.06461	355
5	rs2569254	158751249	IL12B	A	G	36/218	195/833	0.1887	105
5	rs1003199	158755566	IL12B	A	G	113/143	523/505	0.03566	672
5	rs1433048	158755845	IL12B	G	A	65/191	214/814	0.3	59
6	rs909253	31540313	LTA	G	A	75/181	333/695	0.5789	18
6	rs1800630	31542476	TNF	A	C	54/202	163/863	0.1373	152
6	rs1800629	31543031	TNF	A	G	33/215	156/858	0.5	25
6	rs3093662	31544189	TNF	G	A	23/233	85/943	1	6
6	rs4711998	52050353	IL17A	A	G	56/198	261/767	0.5714	20
6	rs4711998	52050353	IL17A	A	G	56/198	261/767	0.5714	20
6	rs8193036	52050493	IL17A	G	A	63/193	268/758	0.6923	12
6	rs8193036	52050493	IL17A	G	A	63/193	268/758	0.6923	12
6	rs3819024	52050786	IL17A	G	A	82/174	320/708	0.5789	18

6	rs3819024	52050786	IL17A	G	A	82/174	320/708	0.5789	18
6	rs3819025	52051274	IL17A	A	G	17/239	53/975	0.3478	45
6	rs3819025	52051274	IL17A	A	G	17/239	53/975	0.3478	45
6	rs7747909	52054249	IL17A	A	G	58/198	231/797	0.6667	14
6	rs7747909	52054249	IL17A	A	G	58/198	231/797	0.6667	14
6	rs1974226	52055335	IL17A	A	G	40/212	184/810	0.4815	26
6	rs1974226	52055335	IL17A	A	G	40/212	184/810	0.4815	26
7	rs1800795	22766645	IL6	G	C	99/155	436/584	0.4054	36
7	rs2069840	22768572	IL6	C	G	98/150	367/641	0.4667	29
7	rs2069861	22771654	IL6	A	G	19/233	106/910	0.2159	87
11	rs5744280	112016514	IL18	A	G	92/162	352/676	0.1615	129
11	rs1834481	112023827	IL18	C	G	61/195	274/754	0.625	15
11	rs5744247	112026156	IL18	C	G	28/228	92/936	0.3148	53
11	rs1945764	118182801	CD3E	G	A	83/173	359/667	0.2687	66
11	rs3782042	118185919	CD3E	A	G	104/152	367/661	0.2111	89
11	rs3181261	118212671	CD3D	A	G	13/243	85/941	0.09167	239
11	rs7947185	118214726	CD3G	G	A	75/181	336/690	0.5714	20
11	rs3212262	118217854	CD3G	A	C	34/222	116/912	0.5789	18
11	rs1561966	118221474	CD3G	A	G	80/172	327/685	0.8571	6
11	rs4544037	118222062	CD3G	G	A	48/208	216/812	0.7778	8
12	rs2069728	68547784	IFNG	A	G	17/239	67/961	1	6
12	rs2069716	68550815	IFNG	G	A	18/236	61/953	0.6923	12
12	rs1861494	68551409	IFNG	G	A	68/188	297/731	0.5455	21
12	rs2069707	68554288	IFNG	C	G	17/239	80/948	0.6923	12
12	rs1179251	68645051	IL22	G	C	20/236	77/951	0.8571	6
12	rs2227491	68646521	IL22	A	G	111/145	445/579	1	6
12	rs2227513	68647339	IL22	G	A	45/211	228/800	0.2571	69
19	rs11466338	41845801	TGFB1	G	A	22/234	63/963	0.1818	109
19	rs4803455	41851509	TGFB1	A	C	129/123	491/535	0.6923	12
19	rs1800469	41860296	TGFB1	A	G	67/183	310/690	0.5455	21
20	rs752118	44746738	CD40	A	G	55/197	237/785	0.7778	8

20	rs11569317	44750850	CD40	C	G	20/236	51/977	0.1019	215
20	rs11569323	44752304	CD40	G	A	27/229	101/927	0.8571	6
20	rs11569334	44756263	CD40	G	A	12/238	56/956	0.8571	6
20	rs3765457	44757213	CD40	G	A	43/213	162/864	0.07256	316
20	rs2143699	44759166	CD40	A	G	21/235	121/901	0.1048	209
23	rs3092936	135736205	CD40LG	G	A	0/0	0/0	1	6
23	rs3092923	135741185	CD40LG	G	A	0/0	0/0	1	6

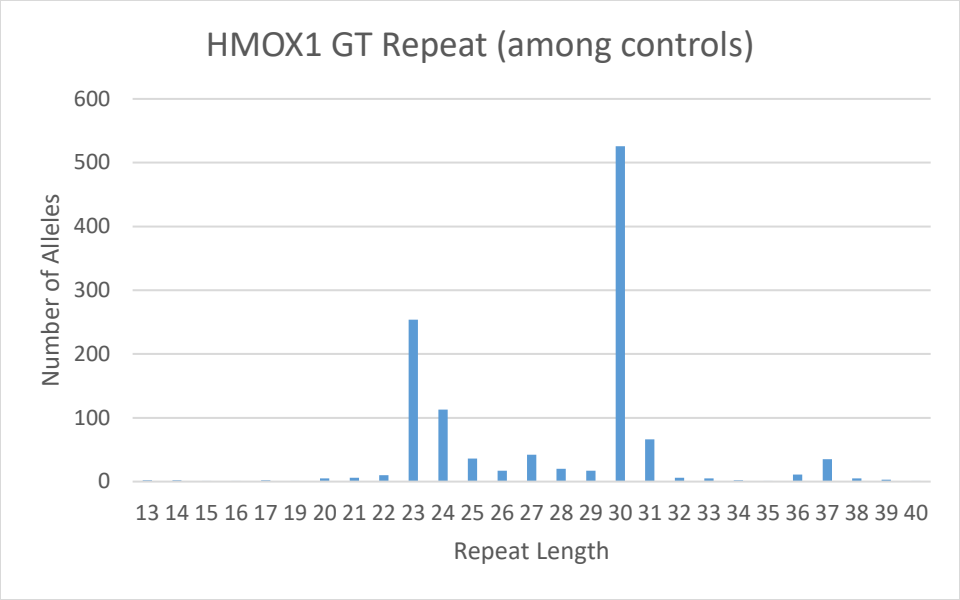
Supplementary Table 4: Permutation results for investigation of association between immune response variants outside the Major Histocompatibility Region and inhibitors among enrollees in the Hemophilia Inhibitor Research Study

	All			Mild			Moderate			Severe			Intron 22 Type 1 Inversion		
	INH (-) N=595	INH (+) N=161	OR (95% CI)	INH (-) N=136	INH (+) N=13	OR (95% CI)	INH (-) N=96	INH (+) N=14	OR (95% CI)	INH (-) N=363	INH (+) N=118	OR (95% CI)	INH (-) N=130	INH (+) N=58	OR (95% CI)
HMOX1 Genotype															
SS	86 (14.5)	16 (9.9)	REF	26 (19.1)	2 (15.4)	REF	15 (15.6)	2 (14.3)	REF	45(12.4)	10 (8.5)	REF	15 (11.5)	5 (8.6)	REF
SL	261 (43.9)	67 (41.6)	1.53 (0.85-2.77)	54 (39.7)	10 (76.9)	1.30 (0.25-6.74)	50 (52.1)	11 (78.6)	1.11 (0.21-5.94)	157 (43.3)	44 (37.3)	1.53 (0.72-3.25)	61 (46.9)	25 (43.1)	1.38 (0.45-4.23)
LL	248 (41.7)	78 (48.5)		56 (41.2)	1 (7.7)		31 (32.3)	1 (7.1)		161 (44.4)	64 (54.2)		54 (41.5)	28 (48.3)	

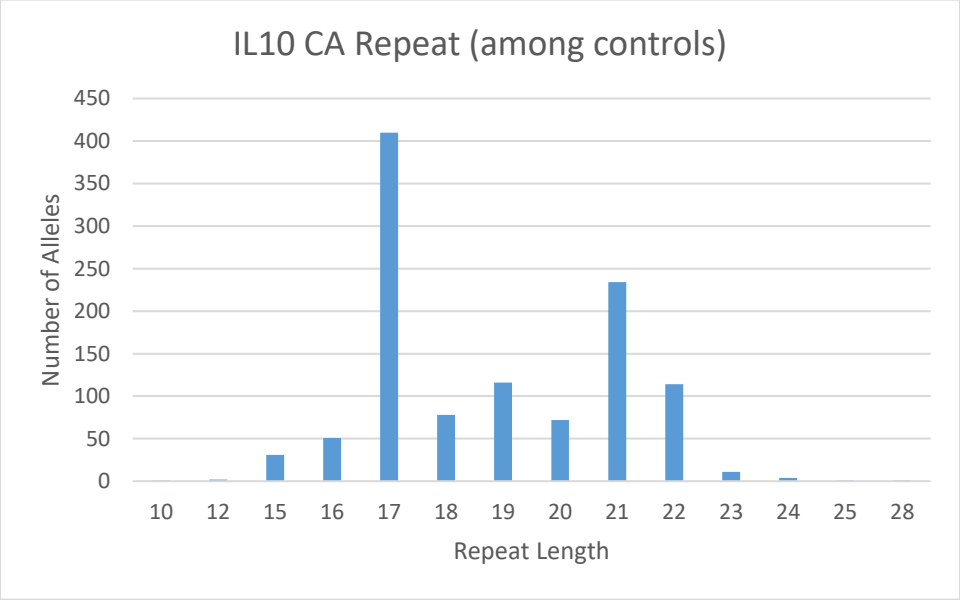
Supplementary Table 5: Association between *HMOX1* $(GT)_n$ and inhibitors, overall, by severity, and among persons with intron 22 Type 1 inversion among enrollees in the Hemophilia Inhibitor Research Study

	All			Mild			Moderate			Severe			Intron 22 Type 1 Inversion		
	INH (-) N=563	INH (+) N=151	OR (95% CI)	INH (-) N=127	INH (+) N=13	OR (95% CI)	INH (-) N=88	INH (+) N=14	OR (95% CI)	INH (-) N=348	INH (+) N=104	OR (95% CI)	INH (-) N=124	INH (+) N=55	OR (95% CI)
IL10 Genotype															
SS	132 (23.5)	48 (31.8)	REF	30 (23.6)	2 (15.4)	REF	15 (17.1)	6 (42.9)	REF	87 (25.0)	36 (34.6)	REF	25 (20.2)	19 (34.6)	REF
SL	309 (54.9)	62 (41.1)	0.66 (0.43-0.99)	71 (55.9)	6 (46.2)	1.7 (0.33-8.78)	49 (55.7)	5 (35.7)	0.27 (0.08-0.96)	189 (54.3)	47 (45.2)	0.69 (0.42-1.12)	75 (60.5)	22 (40.0)	0.48 (0.23-1.01)
LL	122 (21.7)	41 (27.2)		26 (20.5)	5 (38.5)		24 (27.3)	3 (21.4)		72 (20.7)	21 (20.2)		24 (19.4)	14 (25.5)	

Supplementary Table 6: Association between *IL10* $(CA)_n$ and inhibitors, overall, by severity, and among persons with intron 22 Type 1 inversion among enrollees in the Hemophilia Inhibitor Research Study



Supplementary Figure 1: Distribution of *HMOX1* (GT)n repeat length among enrollees in the Hemophilia Inhibitor Research Study without inhibitors



Supplementary Figure 1: Distribution of *IL10* (CA)n repeat length among enrollees in the Hemophilia Inhibitor Research Study without inhibitors

Chapter 6

Component	Beta
HLA-DQB1*06	0.173953
HLA-DRB1*15	0.494696
rs1800629 (AG/AA vs GG)	0.223144
rs1800896 (AG/AA vs GG)	-0.30111
IL10 RPT Length (NOT 22 vs 22)	-0.54473

Supplementary Table 1: Weights of genotypes included in meta-analysis-based score.

Component	Beta
IL10 RPT Length (S: <=18, L: >18; any L vs SS)	-0.4199
rs17561 (AC/AA vs CC)	-0.5425
rs1003199 (GA/AA vs GG)	-0.5139
rs16829984 (CG/CC vs GG)	0.5495
HLA-DPB1	
*04	0.9234
*13	1.4246
*17	1.6635
*19	1.7405

Supplementary Table 2: Weights of genotypes included in HIRS-based score.