Robust, flexible, and scalable tests for Hardy-Weinberg Equilibrium across diverse ancestries

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ABSTRACT

Traditional Hardy-Weinberg equilibrium (HWE) tests (the χ^2 test and the exact test) have long been used as a metric for evaluating genotype quality, as technical artifacts leading to incorrect genotype calls often can be identified as deviations from HWE. However, in datasets comprised of individuals from diverse ancestries, HWE can be violated even without genotyping error, complicating the use of HWE testing to assess genotype data quality. In this manuscript, we present the Robust Unified Test for HWE (RUTH) to test for HWE while accounting for population structure and genotype uncertainty, and evaluate the impact of population heterogeneity and genotype uncertainty on the standard HWE tests and alternative methods using simulated and real sequence datasets. Our results demonstrate that ignoring population structure or genotype uncertainty in HWE tests can inflate false positive rates by many orders of magnitude. Our evaluations demonstrate different tradeoffs between false positives and statistical power across the methods, with RUTH consistently amongst the best across all evaluations. RUTH is implemented as a practical and scalable software tool to rapidly perform HWE tests across millions of markers and hundreds of thousands of individuals while supporting standard VCF/BCF formats. RUTH is publicly available at https://www.github.com/statgen/ruth.

INTRODUCTION

Hardy-Weinberg equilibrium (HWE) is a fundamental theorem of population genetics and has been one of the key mathematical principles to understand the characteristics of genetic variation in a population for more than a century (HARDY 1908; WEINBERG 1908). HWE describes a remarkably simple relationship between allele frequencies and genotype frequencies which is constant across generations in homogeneous, random-mating populations. Genetic variants in a homogeneous population typically follow HWE except for unusual deviations due to very strong case-control association and enrichment (NIELSEN *et al.* 1998), sex linkage, or non-random sampling (WAPLES 2015).

HWE tests are often used to assess the quality of microsatellite (VAN OOSTERHOUT *et al.* 2004), SNP-array (WIGGINTON *et al.* 2005), and sequence-based (DANECEK *et al.* 2011) genotypes. Testing for HWE may reveal technical artifacts in sequence or genotype data, such as high rates of genotyping error and/or missingness, or sequencing/alignment errors (NIELSEN *et al.* 2011). It can also identify hemizygotes in structural variants which are incorrectly called as homozygotes (MCCARROLL *et al.* 2006). Quality control for array-based or sequence-based genotypes typically includes a HWE test to detect and filter out artifactual or poorly genotyped variants (LAURIE *et al.* 2010; NIELSEN *et al.* 2011).

While HWE tests are commonly and reliably used for variant quality control in samples from homogeneous populations, applying them to more diverse samples remains challenging. When analyzing individuals from a heterogeneous population, the standard HWE tests may falsely flag real, well-genotyped variants, unnecessarily filtering them out for downstream analyses (HAO AND STOREY 2019). This problem is important since genetic studies increasingly

collect genetic data from heterogeneous populations. In principle, HWE tests in these structured populations can be performed on smaller cohorts with homogenous backgrounds (BYCROFT *et al.* 2018), and the test statistics combined using Fisher's or Stouffer's method (MOSTELLER AND FISHER 1948; STOUFFER 1949). However, such a procedure requires much more effort than using a single HWE test across all samples and information that may be imperfect or unavailable.

Here, we describe RUTH (Robust Unified Test for Hardy-Weinberg Equilibrium) which tests for HWE under heterogeneous population structure. Our primary motivation for developing RUTH is to robustly filter out artifactual or poorly genotyped variants using HWE test statistics. RUTH is (1) computationally efficient, (2) robust against various degrees of population structure, and (3) flexible in accepting key representations of sequence-based genotypes including best-guess genotypes and genotype likelihoods. We perform systematic evaluations of RUTH and alternative methods for HWE testing using simulated and real data to explore the advantages and disadvantages of these methods for samples of diverse ancestries.

MATERIALS AND METHODS

Unadjusted HWE tests

Consider a study of n participants with true (unobserved) genotypes $g_1, g_2, \cdots, g_n$ at a bi-allelic variant coded as 0 (reference homozygote), 1 (heterozygote), or 2 (alternate homozygote). Represent the best-guess/hard-call (observed) genotypes as $\hat{g}_1, \hat{g}_2, \cdots, \hat{g}_n$. A simple HWE test uses the chi-squared statistic to compare the expected and observed genotype counts assuming no population structure and no genotype uncertainty. The chi-squared HWE test

statistic is defined as $T_{\chi^2} = \sum_{k=0}^{2} \frac{(c_k - \hat{c}_k)^2}{\hat{c}_k}$ where $c_j = \sum_{i=0}^{n} I(\hat{g}_i = j)$ (ignoring missing genotypes), $\hat{p} = \frac{c_1 + 2c_2}{2n}$, $\hat{q} = 1 - \hat{p}$, $\hat{c}_0 = n\hat{q}^2, \hat{c}_1 = 2n\hat{p}\hat{q}$, and $\hat{c}_2 = n\hat{p}^2$. Under HWE, the asymptotic distribution of T_{χ^2} is usually assumed to follow χ_1^2 (Rohlfs and Weir 2008). An exact test is known to be more accurate for finite samples, particularly for rare variants (Wigginton *et al.* 2005). HWE tests stratified by case-control status are known to prevent an inflation of Type I errors for disease-associated variants (Li and Li 2008). Widely used software tools such as PLINK (Purcell *et al.* 2007) and VCFTools (Danecek *et al.* 2011) implement an exact HWE test based on best-guess genotypes. We will refer to the exact test as the unadjusted test.

Existing HWE tests accounting for structured populations

The unadjusted HWE test assumes that the population is homogeneous. If a study is comprised of a set of discrete structured subpopulations, a straightforward extension of the unadjusted test is to (1) stratify each study participant into exactly one of the subpopulations, (2) perform the unadjusted HWE test for each subpopulation separately, and (3) meta-analyze test statistics across subpopulations to obtain a combined p-value using Stouffer's method (Stouffer *et al.* 1949). More specifically, let $z_1, z_2, \cdots, z_s$ be the z-scores from HWE test statistics for *s* distinct subpopulations with sample sizes $n_1, n_2, \cdots, n_s$. A combined meta-analysis HWE test statistic across the subpopulations is then $T_{meta} = \frac{\sum_{i=1}^{s} z_i \sqrt{n_i}}{\sqrt{\sum_{i=1}^{s} n_i}}$, which asymptotically follows a standard normal distribution when each subpopulation follows HWE.

When the population cannot be easily stratified into distinct subpopulations (e.g. intra-continental diversity or an admixed population), a quantitative representation of genetic

ancestry, such as principal component (PC) coordinates or fractional mixture over subpopulations, can be more useful for representing each study participant's genetic diversity (ROSENBERG *et al.* 2002; PRICE *et al.* 2006). HWES takes PCs as additional input to perform HWE tests under population structure with logistic regression (SHA AND ZHANG 2011), and a similar idea was suggested by Hao and colleagues (2016). However, existing implementations do not support sequence-based genotypes (where genotype uncertainty may remain at low or moderate sequencing depth) or other commonly used formats for genetic array data. A recent method, PCAngsd estimates PCs from uncertain genotypes represented as genotype likelihoods (MEISNER AND ALBRECHTSEN 2019) and uses these estimates to perform a likelihood ratio test (LRT) for HWE, which is similar to the LRT version of RUTH with differences in computational performance (see below).

Robust HWE testing with RUTH

Here we describe RUTH (Robust and Unified Test for Hardy-Weinberg equilibrium) to enable HWE testing under structured populations, which is especially useful for large sequencing studies. We developed RUTH to produce HWE test statistics to allow quality control of sequence-based variant callsets from increasingly diverse samples. RUTH models the uncertainty encoded in sequence-based genotypes to robustly distinguish true and artifactual variants in the presence of population structure, and seamlessly scales to millions of individuals and genetic variants.

We assume the observed genotype for individual i can be represented as a genotype likelihood (GL) $L_i^{(G)} = \Pr(Data_i | g_i = G)$, where $Data_i$ represents observed data (e.g. sequence or array), and $g_i \in \{0,1,2\}$ the true (unobserved) genotype. For example, GLs for

sequence-based genotypes can be represented as $L_i^{(G)} = \prod_{j=1}^{d_i} \Pr(r_{ij}|g_i = G; q_{ij})$ where d_i is the sequencing depth, r_{ij} is the observed read, and q_{ij} is the corresponding quality score (EWING AND GREEN 1998; JUN *et al.* 2012). We model GLs for best-guess genotypes $\hat{g}_i$ from SNP arrays as $L_i^{(G)} = (1-e_i)^2,\ 2e_i(1-e_i),\ e_i^2$ for $\hat{g}_i = 2, 1, 0$ where e_i is assumed per-allele error rate. Imputed genotypes may also be approximately modeled using this framework, but the current implementation requires creating a pseudo-genotype likelihood to describe this uncertainty (see Discussion).

Accounting for Population Structure with Individual-Specific Allele Frequencies

We account for population structure by modeling individual-specific allele frequencies from quantitative coordinates of genetic ancestry such as PCs, similar to the model (HAO *et al.* 2016). For any given variant, instead of assuming that genotypes follow HWE with a single universal allele frequency across all individuals, we assume that genotypes follow HWE with heterogeneous allele frequencies specific to each individual, modeled as a function of genetic ancestry. Let $\boldsymbol{x_i} \in \mathbb{R}^k$ represent the genetic ancestry of individual i, where k is the number of PCs used. We estimate individual-specific allele frequency p as a bounded linear function of genetic ancestry

$$p(\boldsymbol{x}_i; \boldsymbol{\beta}) = \begin{cases} \boldsymbol{\beta}^T \boldsymbol{x}_i & \varepsilon \leq \boldsymbol{\beta}^T \boldsymbol{x}_i \leq 1-\varepsilon \\ \varepsilon & \boldsymbol{\beta}^T \boldsymbol{x}_i < \varepsilon \\ 1-\varepsilon & \boldsymbol{\beta}^T \boldsymbol{x}_i > 1-\varepsilon \end{cases},$$

where ε is the minimum frequency threshold. We used $\varepsilon = \frac{1}{4n}$ in our evaluation. Even though we used a linear model for $p(\boldsymbol{x}_i; \boldsymbol{\beta})$ for computational efficiency, it is straightforward to apply a logistic model, which is arguably better (YANG *et al.* 2012; HAO *et al.* 2016).

Let $p_i = p(\boldsymbol{x}_i; \boldsymbol{\beta})$ and $q_i = 1 - p_i$ be the individual specific allele frequencies of the non-reference and reference alleles for individual i. Under the null hypothesis of HWE, the frequencies of genotypes (0, 1, 2) are $[q_i^2,\ 2p_iq_i,\ p_i^2]$. Under the alternative hypothesis, we assume these frequencies are $[q_i^2 + \theta p_i q_i,\ 2p_iq_i(1-\theta),\ p_i^2 + \theta p_i q_i]$ where θ is the inbreeding coefficient. This model is a straightforward extension of a fully general model where p_i, q_i is identical across all samples. Then the log-likelihood across all study participants is

$$l(\boldsymbol{\beta}, \theta) = \sum_{i=1}^{n} \log\left[L_i^{(0)}(q_i^2 + \theta p_i q_i) + L_i^{(1)}\, 2p_iq_i(1-\theta) + L_i^{(2)}(p_i^2 + \theta p_i q_i)\right]$$

Under both the null ($\theta = 0$) and alternative ($\theta \neq 0$) hypotheses, we maximize the log-likelihood using an Expectation-Maximization (E-M) algorithm (DEMPSTER *et al.* 1977). As we empirically observed quick convergence within several iterations in most cases, we used a fixed (n=20) number of iterations in our implementation.

RUTH Score Test

The score function of the log-likelihood is

$$U(\theta) = \sum_{i=1}^{n} \frac{p_iq_i\left[L_i^{(0)} - 2L_i^{(1)} + L_i^{(2)}\right]}{L_i^{(0)}(q_i^2 + \theta p_i q_i) + L_i^{(1)}\ 2p_iq_i(1-\theta) + L_i^{(2)}(p_i^2 + \theta p_i q_i)} = \sum_{i=1}^{n} u_i(\theta)$$

Since $u_i'(\theta) = -u_i^2(\theta)$, we construct a score test statistic of H_0: $\theta = 0$ vs H_1: $\theta \neq 0$ as:

$$T_{score} = \frac{[U(0)]^2}{I(0)} = \frac{[\sum_{i=1}^{n} u_i(0)]^2}{\sum_{i=1}^{n} u_i^2(0)}$$

where $I(0)$ is the Fisher information under the null hypothesis. Under the null, T_{score} has an asymptotic chi-squared distribution with one degree of freedom, i.e. $T_{score} \sim \chi_1^2$. We estimate $\widehat{\boldsymbol{\beta}}$ with an E-M algorithm.

RUTH Likelihood Ratio Test

The log-likelihood function $l(\boldsymbol{\beta}, \theta)$ can also be used to calculate a likelihood ratio test statistic:

$$T_{LRT} = 2\left[\max_{\boldsymbol{\beta},\theta} l(\boldsymbol{\beta}, \theta) - \max_{\boldsymbol{\beta}} l(\boldsymbol{\beta}, 0)\right].$$

Like the score test, we estimate MLE parameters $\boldsymbol{\beta}, \theta$ iteratively using an E-M algorithm to test $H_0: \theta = 0$ vs $H_1: \theta \neq 0$. Under the null hypothesis, the asymptotic distribution of T_{LRT} is expected to follow χ_1^2. This test is very similar to the likelihood-ratio test proposed by PCAngsd (Meisner and Albrechtsen 2019), except PCAngsd does not re-estimate $\boldsymbol{\beta}$ under the alternative hypothesis. In principle, the RUTH LRT should be slightly more powerful due to this difference; we expect the practical difference in power to be small, as deviations from HWE usually do not change the estimates of $\boldsymbol{\beta}$ substantially.

Simulation of genotypes and sequence reads under population structure

We simulated sequence-based genotypes under population structure using the following procedure. First, for each variant, we simulated an ancestral allele frequency and population-specific allele frequencies. Second, we sampled unobserved (true) genotypes based on these allele frequencies. Third, we sampled sequence reads based on the unobserved genotypes. Fourth, we generated genotype likelihoods and best-guess genotypes based on sequence reads.

To simulate ancestral and population-specific allele frequencies, we followed the BALDING AND NICHOLS (1995) procedure, except we sampled ancestral allele frequencies from $p \sim Uniform(0,1)$ instead of $p \sim \mathrm{Uniform}(0.1, 0.9)$ to include rare variants. For each of $K \in \{1, 2, 5, 10\}$ populations, we sampled population-specific allele frequencies from $p_k \sim Beta\left(\frac{p(1-F_{st})}{F_{st}}, \frac{(1-p)(1-F_{st})}{F_{st}}\right)$, where $k \in \{1, \cdots, K\}$, and $F_{st} \in \{.01, .02, .03, .05, .10\}$ was the fixation index to quantify the differentiation between the populations, as suggested by Holsinger (HOLSINGER 1999) and implemented in previous studies (HOLSINGER *et al.* 2002; BALDING 2003). Because p_k no longer follows the uniform distribution, we used rejection sampling to ensure that $\bar{p} = \frac{1}{K}\sum_{k=1}^{K} p_k$ is uniformly distributed across 100 bins across simulations to avoid artifacts caused by systematic differences in allele frequencies.

The unobserved genotype $G_i \in \{0,1,2\}$ for individual $i \in \{1, \cdots, n_k\}$, belonging to population k with sample size n_k, was simulated from genotype frequencies $(q_k^2 + \theta\, p_k q_k, 2p_k q_k(1-\theta), p_k^2 + \theta\, p_k q_k)$, where $q_k = 1 - p_k$ and $\theta \in \left[-\min\left(\frac{q_k}{p_k}, \frac{p_k}{q_k}\right), 1\right]$ quantifies deviation from HWE; $\theta = 0$ represents HWE, while $\theta < 0$ and $\theta > 0$ represent excess heterozygosity and homozygosity compared to HWE expectation, respectively. In our experiments, we evaluated $\theta \in \{0, \pm.01, \pm.05, \pm.1, \pm.5\}$. When θ was smaller than the minimum possible value for a specific population, we replaced it with the minimum value.

We simulated sequence reads based on unobserved genotypes, sequence depths, and base call error rates. To reflect the variation of sequence depths between individuals, we simulated the mean depth of each sequenced sample to be distributed as $\mu_i \sim Uniform(1, 2D-1)$, where D is the expected depth and $D = 5$ and $D = 30$ representing low-coverage and deep sequencing, respectively. For each sequenced sample and variant site,

we sampled the sequence depth from $d_i \sim Poisson(\mu_i)$. Each sequence read carried either of the possible unobserved (true) alleles $r_{ij} \in \{0,1\}$, where $j \in \{1, \cdots, d_i\}$. Given unobserved genotype G_i, we generated $r_{ij} \sim Bernoulli\left(\frac{G_i}{2}\right)$, with observed allele $o_{ij} = (1 - e_{ij})r_{ij} + e_{ij}(1 - r_{ij})$ flipping to the other allele when a sequencing error occurs with probability $e_{ij} \sim Bernoulli(\epsilon)$. We used $\epsilon = 0.01$ throughout our simulations (which corresponds to phred-scale base quality of 20) and assumed that all base calling errors switched between reference and alternate alleles.

We then generated genotype likelihoods and best-guess genotypes from the simulated alleles. Let $t_i = \sum_{j=1}^{d_i} o_{ij}$ be the observed alternate allele count. The GLs for the three possible genotypes are $L_i^{(0)} = (1 - \epsilon)^{d_i - t_i}(\epsilon)^{t_i}$, $L_v^{(1)} = 0.5^{d_i}$, $L_i^{(2)} = (\epsilon)^{d_i - t_i}(1 - \epsilon)^{t_i}$. We called best-guess genotypes by using the overall ancestral allele frequency $\bar{p}$ for a given variant as the prior, then calling the genotype corresponding to the highest posterior probability among $\left(L_i^{(0)}(1 - \bar{p})^2,\ 2L_i^{(1)}\bar{p}(1 - \bar{p})^2,\ L_i^{(2)}\bar{p}^2\right)$ for each sample. For each possible combination of F_{st}, K, and θ, we generated 50,000 independent variants across a set of $n = 5{,}000$ samples with per-ancestry samples sizes $n_k = \frac{n}{K}$.

Evaluation of Type I Error and Statistical Power

We used different p-value thresholds, F_{st} values, number of ancestry groups K, and average sequencing depth D to determine the number of variants significantly deviating from HWE. To evaluate Type I error, we simulated sequence reads under HWE ($\theta = 0$) and calculated the proportion of significant variants at each p-value threshold. In RUTH tests, we assumed PCs were accurately estimated using true genotypes unless indicated otherwise. For real data, we

summarized ancestral information by projecting PCs estimated from their full genomes onto the reference PC space of the Human Genome Diversity Panel (HGDP) (LI *et al.* 2008) using verifyBamID2 (ZHANG *et al.* 2020), similar to the procedure for variant calling in the TOPMed Project, which has already integrated RUTH as part of its quality control pipeline (https://github.com/statgen/topmed_variant_calling).

In all datasets, we evaluated the tradeoff between Type I Error and power for each method using precision-recall curves (PRCs) and receiver-operator characteristic curves (ROCs). In simulated data, we considered variants with $\theta = 0$ to be true negatives and variants with $\theta = -0.05$ to be true positives. In both our 1000G and TOPMed data, we labeled HQ variants as negative and LQ variants as positive.

Data source

To evaluate our method, we used sequence-based genotype data from the 1000 Genomes Project (1000G) (THE 1000 GENOMES PROJECT CONSORTIUM *et al.* 2015) and the Trans-Omics Precision Medicine (TOPMed) Project (TALIUN *et al.* 2019). In both cases, we used a subset of variants from chromosome 20. For 1000G, we started with 1,812,841 variants in 2,504 individuals, with an average depth of $7.0\times$. For TOPMed, we started with 12,983,576 variants in 53,831 individuals, with an average depth of $37.2\times$.

Application to 1000 Genomes data

To test our method on 1000G data, we first needed to define two sets of variants: one set which is expected to follow HWE, and another set which is expected to deviate from HWE. Unlike simulated data, variants in 1000G are not clearly classified into “true” or “artifactual”, so

evaluation of false positives and power is less straightforward. We focused on two subsets of variants in chromosome 20 which serve as proxies for these two variant types. We selected non-monomorphic sites found in both the Illumina Infinium Omni2.5 genotyping array and in HapMap3 (THE INTERNATIONAL HAPMAP CONSORTIUM *et al.* 2010) as "high-quality" (HQ) variants that mostly follow HWE after controlling for ancestry, ending up with 17,740 variants. Similarly, we selected variants that displayed high discordance between duplicates or Mendelian inconsistencies within family members in TOPMed sequencing study as "low quality" (LQ) variants that should be enriched for deviations from HWE even after accounting for ancestry, ending up with 10,966 variants. Among 329,699 LQ variants from TOPMed in chromosome 20, we found that only 10,966 overlap with 1000 Genome samples because likely artifactual variants were stringently filtered prior to haplotype phasing. We suspect that a substantial fraction of these 10,966 LQ variants are true variants since they passed all of the 1000G Project's quality filters. Nevertheless, we still expect a much larger fraction of these LQ variants to deviate from HWE compared to HQ variants.

We evaluated multiple representations of sequence-based genotypes from 1000G. As 1000G samples were sequenced at relatively low-coverage of $7.0\times$ on average, best-guess genotypes inferred only from sequence reads (raw GT) tend to have poor accuracy. Therefore, the officially released best-guess genotypes in 1000G were estimated by combining genotype likelihoods (GL), calculated based on sequence reads, with haplotype information from nearby variants through linkage-disequilibrium (LD)-aware genotype refinement using SHAPEIT2 (DELANEAU *et al.* 2013). This procedure resulted in more accurate genotypes (LD-aware GT), but it implicitly assumed HWE during refinement. As different representations of sequence genotypes may result in different performance in HWE tests, we evaluated all three different

representations - raw GT, LD-aware GT, and GL. In all tests of RUTH using hard genotype calls, we assumed the error rate for GT-based genotypes to be 0.5%, which is representative of a typical non-reference genotype error rate for SNP arrays. We restricted our analyses to biallelic variants. The positions and alleles of 1000G and TOPMed variants were matched using the liftOver software tool (KUHN *et al.* 2013).

We evaluated all tests as described above. For meta-analysis with Stouffer's method, we divided the samples into 5 strata, using the five 1000G super population code labels – African (AFR), Admixed American (AMR), East Asian (EAS), European (EUR), and South Asian (SAS). To obtain PC coordinates for 1000G samples, we estimated 4 PCs from the aligned sequence reads (BAM) with verifyBamID2 (ZHANG *et al.* 2020), using PCs from 936 samples from the Human Genome Diversity Project (HGDP) panel as reference coordinates. The RUTH score test and LRT used these PCs as inputs, along with genotypes in raw GT, LD-aware GT, and GL formats. For PCAngsd, we used GLs from all variants tested as the input. We limited the analysis to a single chromosome due to the heavy computational requirements of PCAngsd.

Application to TOPMed Data

We analyzed variants from 53,831 individuals from the TOPMed sequencing study (TALIUN *et al.* 2019). These samples came from multiple studies from a diverse spectrum of ancestries, leading to substantial population structure. Using the same criteria as our 1000G analysis, we identified 17,524 high-quality variants and 329,699 low-quality variants across chromosome 20. Since TOPMed genomes were deeply sequenced at $37.2\times$ ($\pm 4.5\times$), LD-aware genotype refinement was not necessary to obtain accurate genotypes. Therefore, we used two genotype representations – raw GT and GL – in our evaluations.

Similar to 1000G, for best-guess genotypes (raw GT), we used PLINK for the unadjusted test. For meta-analysis, we assigned each sample to one of the five 1000G super populations as follows. First, we summarized the genetic ancestries of aligned sequenced genomes with verifyBamID2 by estimating 4 PCs using HGDP as reference. Second, we used Procrustes analysis (DRYDEN AND MARDIA 1998; WANG *et al.* 2010) to align the PC coordinates of HGDP panels (to account for different genome builds) so that the PC coordinates were compatible between TOPMed and 1000G samples. Third, for each TOPMed sample, we identified the 10 closest corresponding individuals from 1000G using the first 4 PC coordinates with a weighted voting system (assigning the closest individual a score of 10, next closest a score of 9, and so on until the 10th closest individual is assigned a score of 1, then adding up the scores for each super population) to determine the super population code that had the highest sum of scores, and therefore best described that sample. In this way, we classified 15,580 samples as AFR, 4,836 as AMR, 29,943 as EUR, 2,960 as EAS, and 716 as SAS. Among these samples, 94.5% had the same super population code for all 10 nearest 1000G neighbors. To evaluate the RUTH score test and LRT for both raw GT and GL, we used 4 PCs estimated by verifyBamID2 (ZHANG *et al.* 2020), consistent with the method applied for the 1000G data.

Impact of Ancestry Estimates on Adjusted HWE Tests

We examined the effect of changing the number of PCs used as input for RUTH tests by using 2 PCs as opposed to 4 PCs. We also evaluated the impact of using different approaches to classify ancestry when adjusting for population structure with meta-analysis. By default, our analysis classified the 1000 Genomes subjects into 5 continental super populations based on published information (THE 1000 GENOMES PROJECT CONSORTIUM *et al.* 2015). For TOPMed, the best-matching

1000 Genomes continental ancestry was carefully determined using the PCA-based matching strategy described above. However, in practice, ancestry classification may be performed with a coarser resolution (JIN *et al.* 2019). To mimic such a setting, we used k-means clustering on the first 2 PCs of our samples to divide individuals into 3 distinct groups, and performed meta-analyses based on this coarse classification for both 1000G and TOPMed data.

Software and data availability

RUTH is available at https://github.com/statgen/ruth. Genotype data from 1000G is available from the International Genome Sample Resource at https://www.internationalgenome.org. TOPMed data is available via a dbGaP application for controlled-access data (see https://www.nhlbiwgs.org for details).

RESULTS

Simulation: Effect of Genotype Uncertainty

To evaluate the impact of genotype uncertainty, we first compared tests in the absence of population structure (i.e. single ancestry). For the unadjusted test, we used only best-guess genotypes (GTs). For PCAngsd, we used only genotype likelihoods (GLs). For RUTH score and likelihood ratio tests, we used both.

Using GLs over GTs substantially reduced Type I errors in HWE tests, especially in low-coverage data (Figure 1A-C). For example, the standard HWE test based on GTs resulted in a 229-fold inflation (22.9%) at $p < .001$ (Figure 1B, Table S1), a threshold which allows the evaluation of Type I error with reasonable precision with 50,000 variants (50 expected false

positives under the null). GT-based RUTH-Score and RUTH-LRT tests showed similar inflation. When GLs were used instead of best-guess genotypes, RUTH-Score and RUTH-LRT had Type I errors close to the null expectation (.001 for RUTH-Score and .0012 for RUTH-LRT). PCAngsd, which also accounts for genotype uncertainty (MEISNER AND ALBRECHTSEN 2019), had similar performance. The severely inflated Type I errors with best-guess genotypes can largely be attributed to high uncertainty and bias towards homozygote reference genotypes in single site calls from low-coverage sequence data, resulting in apparent deviations from HWE. For high-coverage sequence data, inflation of Type I error with GTs was substantially attenuated; inflation nearly disappeared when using GLs (.004 for RUTH-Score and .002 for RUTH-LRT; Figure 1D-F).

Next, we evaluated the power to identify variants truly deviating from HWE at various levels of inbreeding coefficient (θ). For low-coverage sequence data, we skip interpretation of power of GT-based tests owing to their extremely inflated false positive rates. All GL-based tests behaved similarly, achieving ~19-21% power at $p < .001$ with moderate excess heterozygosity ($\theta = -0.05$) (Figure 2B, Table S1). For high-coverage sequence data, the power of GL-based tests at the same p-value threshold increased to ~56-60%, comparable to corresponding GT-based tests. Interestingly, the unadjusted GT-based test showed much lower power than RUTH and PCAngsd tests under excess heterozygosity ($\theta < 0$) while demonstrating much higher power with excess homozygosity ($\theta > 0$). Upon further investigation, we observed that the tests behave very differently for rare variants for which an asymptotic approximation performs poorly.

We also generated precision-recall curves (PRC) and receiver-operator characteristic (ROC) curves to better understand the tradeoff between the Type I errors and power under moderate excess heterozygosity (θ = -.05) (Figure S1C-D). Again, accounting for genotype uncertainty resulted in better empirical power and Type I error, especially for low-coverage data, for which, at an empirical false positive rate of 1%, GL-based tests had 41-45% power, as opposed to 4-10% for GT-based tests. For high-coverage data, GL-based tests had 1-2% greater power than GT-based tests at the same false positive rate. These results suggest that ignoring genotype uncertainty in HWE tests is reasonable for high-coverage sequence data.

Simulation: Impact of Population Structure on HWE Test Statistics

As expected, the unadjusted HWE test had substantially inflated Type I errors under population structure based on the Balding-Nichols (1995) model (Figure 1, Table S1). Even for an intra-continental level of population differentiation (F_{ST} = .01), the Type I errors at $p < .001$ were inflated 13.5-fold even for high-coverage data. With an inter-continental level of differentiation (F_{ST} = .1), we observed orders of magnitude more Type I errors across different simulation conditions. This inflation is expected to increase with larger sample sizes, suggesting that adjustment for population structure is important even if a study focuses on a single continental population.

One simple approach to account for population structure is to stratify individuals into distinct subpopulations to apply HWE tests separately (Bycroft *et al.* 2018), and meta-analyze the results (Figure 3B). Type I errors were appropriately controlled with this approach in high-coverage but not low-coverage data, likely due to unmodeled genotype uncertainty (Figure 1, Table S1). Instead of classifying individuals into distinct subpopulations, RUTH incorporates PCs

to jointly perform HWE tests (Figure 3C). For both low- or high-coverage data, GL-based RUTH tests and PCAngsd showed well-controlled Type I errors, while GT-based tests showed slight (high-coverage) or severe (low-coverage) inflation.

Although meta-analysis resulted in well-controlled Type I errors for high-coverage data, it was considerably less powerful than RUTH. For example, with moderate excess heterozygosity ($\theta = -.05$) across five ancestries ($F_{ST} = .1$), RUTH tests identified 20-27% more variants as significant at $p < .001$ (Figure 2, Table S1) compared to meta-analysis. PRCs also clearly showed better operating characteristics for RUTH and PCAngsd compared to meta-analysis (Figure S2). For example, at an empirical false positive rate of 1%, RUTH showed much greater power (66-68%) than meta-analysis (43%), even though the simulation scenario favors meta-analysis because samples were perfectly classified into distinct subpopulations.

Application to 1000 Genomes WGS data

Next, we evaluated the performance of various HWE tests in low-coverage (~6x) sequence data from the 1000 Genomes Project. We evaluated three representations of genotypes - (1) raw GT, (2) LD-aware GT, and (3) GL, as described in Materials and Methods. Among chromosome 20 variants, we selected 17,740 high-quality (HQ) variants that are polymorphic in GWAS arrays, and 10,966 low-quality (LQ) variants enriched for genotype discordance in duplicates and trios. Unlike simulation studies, not all LQ variants are necessarily expected to violate HWE, so we consider the proportion of significant LQ variants as a lower bound on the sensitivity to identify significant variants. Similarly, not all HQ variants are necessarily expected to follow HWE, although we expect most to do so, so that the proportion of significant HQ variants serves as an upper bound for the false positive rate.

Consistent with our simulation results, all tests based on raw GTs generated from low-coverage sequence data had severe inflation of false positives (Figure 4A, Table 1). This was true even for HQ variants, presumably due to genotyping errors and bias in raw GTs. Standard HWE tests, which model neither genotype uncertainty nor population structure, showed the highest inflation of false positives at 44% for $p < 10^{-6}$, a threshold commonly used for HWE testing in large genetic studies (LOCKE *et al.* 2015; FRITSCHE *et al.* 2016). Modeling population structure substantially reduced inflation, with RUTH tests showing fewer false positives (0.7-1.0% at $p < 10^{-6}$) than meta-analysis (2.0% at $p < 10^{-6}$). False positives were inflated across all methods when using raw GTs.

Consistent with our simulation studies, GL-based RUTH tests reduced false positives even further (0.034% at $p < 10^{-6}$). In contrast to our simulations, PCAngsd demonstrated considerably higher false positives than RUTH (2.1% at $p < 10^{-6}$), likely because PCAngsd estimates PCs from the input data without the ability to use externally provided PCs (see Discussion). The sensitivity for detecting significant LQ variants was also consistent with our simulations (Figure 4B, Table 1). GL-based tests, which showed better control of false positives, identified 22-25% of LQ variants as significant at $p < 10^{-6}$.

Strikingly, while using LD-aware GTs reduced false positives with adjusted tests, it was at the expense of substantially reduced sensitivity to detect LQ variants. The false positive rates of any adjusted test with LD-aware GTs were uniformly lower than those of any GL- and raw GT-based tests across all p-value thresholds (Figure 4A). However, sensitivity was also substantially reduced with LD-aware genotypes (Figure 4B). For example, at $p < 10^{-6}$, GL-based RUTH tests identified 22-23% of LQ variants significant, while using LD-aware GTs halved the proportions.

Running meta-analysis with LD-aware GTs reduced sensitivity even further, likely because the implicit HWE assumption in the LD-aware genotype refinement algorithms may have further reduced false positives and sensitivity by altering the LD-aware genotypes to conform to HWE.

We evaluated PRCs between HQ and LQ variants to further evaluate this tradeoff. The results clearly demonstrated that HWE tests using LD-aware GTs are substantially less robust than tests on other genotype representations (Table S2, Figure S3A). For example, for the RUTH score test, when LD-aware GTs identified 0.1% of HQ variants as significant, 17% of LQ variants were identified as significant. However, with raw GT and GL, 24~27% were identified as significant at the same threshold. Even fewer were significant in meta-analysis with LD-aware GTs (13%). Similar trends were observed across all thresholds, suggesting that using LD-aware GTs results in substantially poorer operating characteristics than other genotype representations. As more accurate genotyping in LD-aware genotype refinement is expected to improve the performance of QC metrics compared to raw GTs, these results are quite striking, and highlight a potential oversight in using LD-aware genotypes in various QC metrics for sequence-based genotypes.

Application to TOPMed Deep WGS data

We evaluated the various HWE tests on a subset of the Freeze 5 variant calls from the high-coverage (~37×) whole genome sequence (WGS) data in the TOPMed Project (TALIUN *et al.* 2019). We identified 17,524 HQ variants and 329,699 LQ variants using the same criteria used for 1000G variants and evaluated raw GTs and GLs. We did not evaluate PCAngsd due to excessive computational time (see “Computational cost” below).

We first evaluated the false positive rates of different HWE tests indirectly by using HQ variants. With a >20-fold larger sample size than 1000G, we identified more significant HQ variants, while the false positive rates were still reasonable with adjusted tests. At $p < 10^{-6}$, 74% of HQ variants were significant with unadjusted tests, while the adjusted GL-based tests identified ~0.3% at $p < 10^{-6}$ (Figure 4C-D, Table 2). Adjusted GT-based tests had only slightly higher levels of false positives at $p < 10^{-6}$. However, inflation was more noticeable at less stringent p-value thresholds suggesting that GL-based tests may be needed for larger sample sizes.

Next, we evaluated the proportions of LQ variants found to be significant by different tests to indirectly evaluate their statistical power. GT- and GL-based RUTH tests showed similar power, while meta-analysis showed considerably lower power. For example, at $p < 10^{-6}$, meta-analysis identified 47% of LQ variants as significant, while RUTH tests identified 54-58%. This pattern was similar across different p-value thresholds (Figure 4C-D) or choices of LQ variants (Table S3, Figure S4). Our results suggest that GL-based RUTH tests are suitable for testing HWE for tens of thousands of deeply sequenced genomes with diverse ancestries, but that using raw GTs will also result in a comparable performance at typically used HWE p-value thresholds (e.g. $p < 10^{-6}$) when performing QC without access to GLs.

We used PRCs to evaluate the tradeoff between empirical false positive rates and power. Consistent with previous results, the GL-based RUTH test showed the best tradeoff between false positives and power, while the GT-based RUTH test and meta-analysis were slightly less robust but largely comparable (Figure S3). Notably, when we evaluated the

different methods at an empirical false positive rate of 0.1%, RUTH score tests had ~4% higher power than RUTH LRT for both raw GTs and GLs (Figure S5-6).

Impact of ancestry estimation accuracy on HWE tests

So far, our evaluations relied on genetic ancestry estimates carefully determined with sophisticated methods (see Materials and Methods). However, simpler approaches may be used instead during the variant QC step, which may affect the performance of adjusted HWE tests. We evaluated whether the number of PC coordinates affected the performance of RUTH tests by comparing the performance of RUTH tests when using 2 PCs to using 4 PCs (default). The results from both simulated and real datasets consistently demonstrated that using 4 PCs led to substantially reduced Type I errors compared to using 2 PCs at a similar level of power (Table S2, Table S4, Figure S7). PRCs also clearly showed that using 4 PCs was more robust against population structure across both simulated and real datasets (Figure S8).

We also evaluated whether the classification accuracy of subpopulations affected the performance of meta-analysis. Instead of assigning 1000 Genomes individuals into five continental populations, we used the k-means algorithm on those samples' top 2 PCs to classify them into 3 crude subpopulations (Figure S9). This led to a much higher false positive rate with virtually no increase in true positives (Figure S10, Table S2). We saw the same pattern in simulated data (Figure S8, Table S5).

Computational cost

We compared the computational costs of RUTH and PCAngsd for simulated and real data. RUTH has linear time complexity to sample size, while PCAngsd appears to have quadratic time

complexity (Tables 3, S6). RUTH also has low memory requirement compared to PCAngsd (for example, 14 MB vs 2 GB for 1000 Genomes data). Extrapolating our results to the whole genome scale, analyzing 1000 Genomes (i.e. 80 million variants) is expected to take 120 CPU-hours for RUTH, and 3,200 CPU-hours for PCAngsd (with >1 TB memory consumption). Additionally, RUTH can be parallelized into smaller regions in a straightforward manner.

DISCUSSION

RUTH is a unified, flexible, and robust approach to incorporate genetic ancestry and genotype uncertainty for testing Hardy-Weinberg Equilibrium capable of handling large amounts of genotype data with structured populations. Sha and Zhang (2011) proposed HWES, an HWE test for structured populations, to address some of these challenges, but it has not been widely used due to the lack of an implementation that supports widely used genotype data formats (e.g. PED, BED, VCF, or BCF) and inability to handle imputed or uncertain genotypes. Hao and colleagues (2016) proposed sHWE which can only handle best-guess (hard call) genotypes (i.e. 0, 1, or 2 for biallelic variants) and does not account for genotype uncertainty. MEISNER AND ALBRECHTSEN (2019) proposed PCAngsd to address some of these issues, but it does not support the standard VCF/BCF formats for sequence-based genotypes, and its current implementation scales poorly with genome-wide analyses of large samples.

Similar to previous studies (SHA AND ZHANG 2011; HAO *et al.* 2016), our proposed framework uses individual-specific allele frequencies rather than allele frequencies pooled across all samples to systematically account for population structure in HWE tests. Unlike previous studies, we model genotype uncertainty in sequence-based genotypes in a likelihood-based framework. We implemented two RUTH tests – a score test and a likelihood ratio test

(LRT) – to test for HWE under population structure for genotypes with uncertainty. While RUTH LRT is similar to the independently developed PCAngsd, the software implementation of RUTH is more flexible, scales much better to large studies, and supports the standard VCF format.

We provide a comprehensive evaluation of various approaches for testing HWE using simulated and real data. Our results demonstrated that modeling population stratification is necessary for HWE tests on heterogenous populations. We showed that accounting for genotype uncertainty via genotype likelihoods performs substantially better than testing HWE with best-guess genotypes, especially for low-coverage sequenced genomes. Importantly, we included the evaluations for an unpublished but commonly used approach – meta-analysis across stratified subpopulations, cohorts, or batches. Our results demonstrate that meta-analysis may be effective in reducing false positives, but at the expense of substantially reduced power compared to RUTH.

We observed that the current implementation of PCAngsd does not scale well to large-scale sequencing data, though in principle it can be implemented more efficiently, because the underlying HWE test itself is similar to RUTH LRT. PCAngsd requires loading all genotypes into memory, which is often infeasible for large sequencing studies. For example, loading all of 1000 Genomes will require ~4.8 TB of memory. In our evaluation of 1000G chromosome 20 variants, the inability of PCAngsd to estimate PCs from the whole genome may have contributed to the observed difference in results from RUTH compared to our simulation studies.

Although our 1000G experiments demonstrated the unexpected result that using raw GTs had better sensitivity than using LD-aware GTs at the same empirical false positive rates for low-coverage data, we do not advocate using raw GTs for low-coverage sequence data. First, the results for raw GTs were still consistently less robust than GL-based RUTH tests. Moreover,

it would be tricky to determine an appropriate p-value threshold when the false positives are severely inflated. Therefore, we strongly advocate using GL-based RUTH tests for robust HWE tests with low-coverage sequence data. For the now more typical high-coverage sequence data, GL-based tests are still preferred, but GT-based RUTH tests should be acceptable for cases in which genotype likelihoods are unavailable.

Our experiment compared using 2 vs 4 PCs only because *verifyBamID2* software tool estimated up to 4 PCs projected onto HGDP panel by default (ZHANG *et al.* 2020). Because our method focuses on testing HWE during the QC steps in sequence-based variant calls, a curated version of PCs, estimated from sequenced cohort themselves, may not be readily available at the time of HWE test. However, it is possible to use a larger number of PCs (e.g. >10 PCs) if available at the time of HWE test. We expect that a larger number of PCs will account for finer-grained population structure and may benefit the performance of HWE test, but additional experiments are needed to quantify the impact of using larger number of PCs.

Our results demonstrate that RUTH score and LRT tests perform similarly in simulated and experimental datasets. Overall, the RUTH-LRT was slightly more powerful than the RUTH-score test at the expense of slightly greater false positive rates, although this tendency was not consistent. We observed that the RUTH tests tended to be slightly more powerful in identifying deviation from HWE in the direction of excess heterozygosity than excess homozygosity when compared to adjusted meta-analysis. These results might be caused by the difference between our model-based asymptotic tests compared to the exact test used in meta-analysis.

We did not evaluate our methods on imputed genotypes in this manuscript. Because imputed genotypes implicitly assume HWE, we suspect that HWE tests based on imputed genotypes may have reduced power compared to directly genotyped variants. It is possible to

use approximate genotype likelihoods instead of best-guess genotypes for imputed genotypes, but this requires genotype probabilities, not just the genotype dosages. If genotype probabilities $\Pr(g_i = G|Data_i)$ are available, they can be converted to genotype likelihoods $L_i^{(G)} = \Pr(Data_i|g_i = G)$ using Bayes' rule by modeling $\Pr(g_i = G)$ as a binomial distribution based on allele frequencies (which implicitly assumes HWE). However, similar to LD-aware genotypes in low-coverage sequencing, the power of HWE tests with imputed genotypes may be poor. Further evaluation is needed to understand how useful this approximation will be compared to alternative methods including the use of best-guess imputed genotypes.

Our methods have room for further improvement. First, we used a truncated linear model for individual-specific allele frequencies for computational efficiency. Although such an approximation was demonstrated to be effective in practice (ZHANG *et al.* 2020), applying a logistic model or some other more sophisticated model may be more effective in improving the precision and recall of RUTH tests. Second, we did not attempt to model or evaluate the effect of admixture in our method. Because HWE is reached in two generations with random mating, accounting for admixed individuals may only have marginal impact. On the other hand, admixture can lead to higher observed heterozygosity. It may be possible to improve RUTH by explicitly modeling and adjusting for the effect of admixture on individual-specific allele frequencies. Systematic evaluations focusing on admixed populations are needed to evaluate RUTH's performance on such samples, and whether an admixture adjustment is necessary. Third, RUTH tests do not account for family structure. We suspect that the apparent inflation of Type I error for the TOPMed data was partially due to sample relatedness. Accounting for family structure in other ways, for example using variance components models, will require much longer computational times and may not be feasible for large-scale datasets. Fourth,

RUTH currently does not directly support imputed genotypes or genotype dosages. In principle, it is possible to convert posterior probabilities for imputed genotypes into genotype likelihoods to account for genotype uncertainty (by using individual-specific allele frequencies). However, because most genotype imputation methods implicitly assume HWE, we suspect that HWE tests on imputed genotypes will be underpowered, similar to our observations with LD-aware genotypes in the 1000 Genomes dataset, even though explicitly modeling posterior probabilities may slightly mitigate this reduction in power.

In summary, we have developed and implemented robust and rapid methods and software tools to enable HWE tests that account for population structure and genotype uncertainty. We performed comprehensive evaluations of both our methods and alternative approaches. Our tools can be used to evaluate variant quality in very large-scale genetic data sets, with the ability to handle standard VCF formats for storing sequence-based genotypes. Our software tools are publicly available at http://github.com/statgen/ruth.

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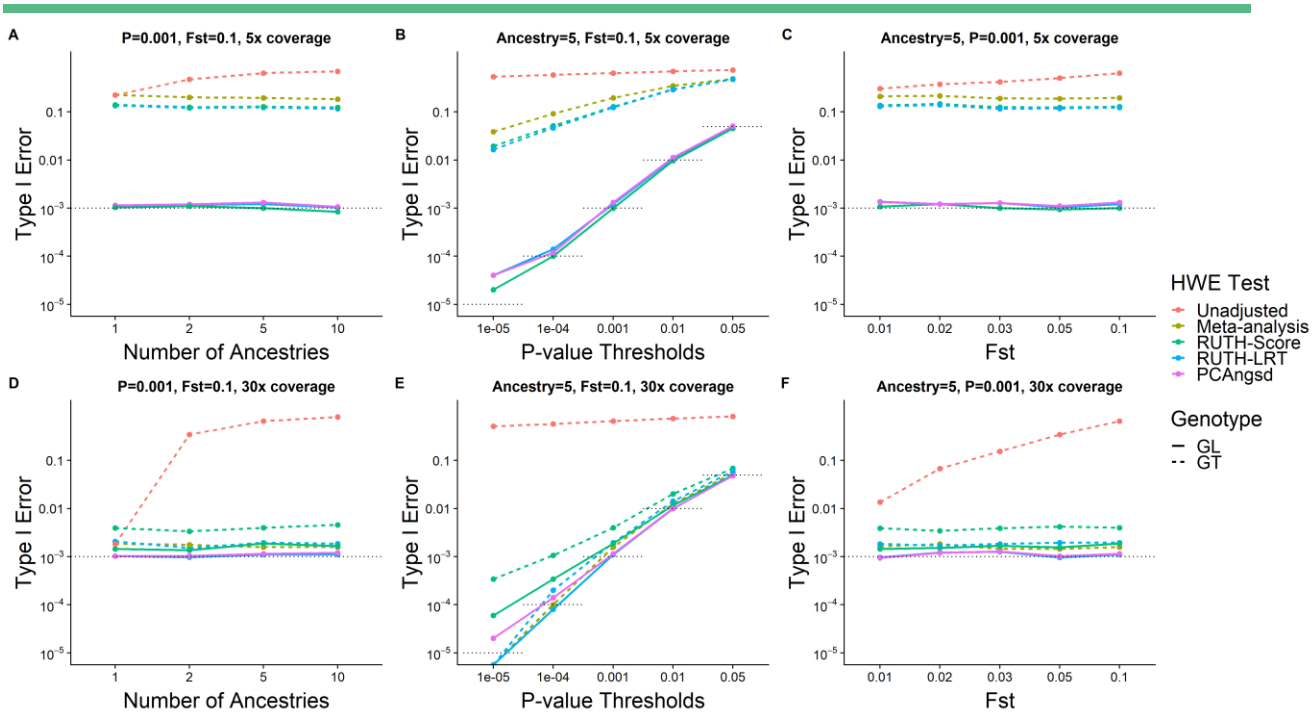

Figure 1

Evaluation of Type I Errors between various HWE tests on simulated genotypes. Under each combination of simulation conditions (number of ancestries, sequencing coverage, and fixation index), we simulated 5,000 samples with 50,000 variants that follow HWE within each of the subpopulations and determined the Type I error performances of different HWE tests based on the proportion of variants labeled as having significant p-values. Five HWE tests – (1) Unadjusted HWE test (WIGGINTON *et al.* 2005) implemented in PLINK-1.9 (PURCELL *et al.* 2007) using hard genotypes, (2) meta-analysis using Stouffer's method across ancestries using hard genotypes (GT), (3) RUTH test using hard genotypes, (4) RUTH test using phred-scale likelihood (GL) computed from simulated sequence reads, and (5) PCAngsd (MEISNER AND ALBRECHTSEN 2019) – were tested under HWE with various parameter settings. Gray dotted lines indicate targeted Type I Error rates. Top panels (A-C) represent results from shallow sequencing (5x), and the bottom panels (D-F) represent results from deep sequencing (30x). Using GL-based genotypes resulted in Type I Error rates closer to the targeted rate than using GT-based genotypes across different numbers of ancestries (A, D), P-value thresholds (B, E), and fixation indices (C, F). The difference is especially large for low-coverage genotypes.

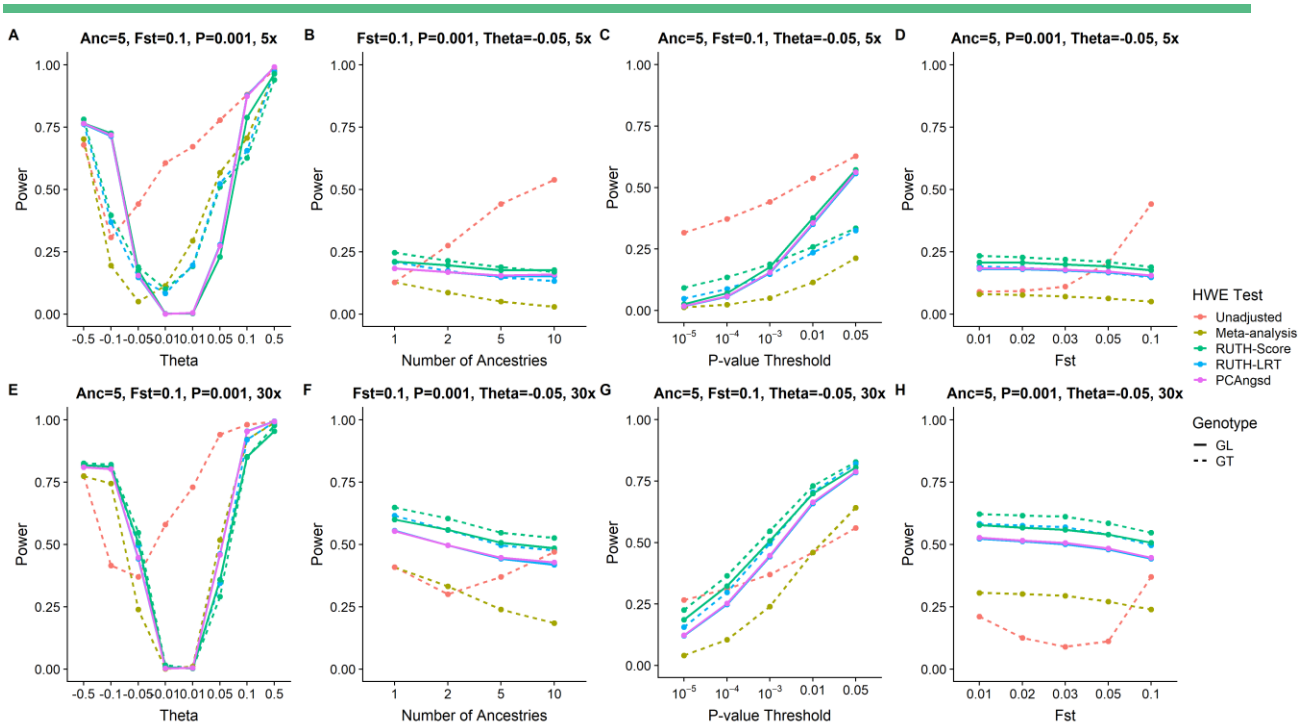

Figure 2

Evaluation of power between different HWE tests on simulated genotypes. Under each combination of simulation conditions (number of ancestries, sequencing coverage, fixation index, and deviation from HWE), we simulated 50,000 variants for 5,000 samples and evaluated the ability of different HWE tests to find the variants significant. Unless otherwise specified, the default simulation parameters are 5 ancestries, with F_{ST}=.1, P-value threshold=.001, and Theta=-0.05. Tests that can find a larger proportion of significant variants are considered more powerful. Five HWE tests – (1) Unadjusted HWE test (WIGGINTON *et al.* 2005) implemented in PLINK-1.9 using hard genotypes (2) RUTH test using hard genotypes, (3) RUTH test using phred-scale likelihood (PL) computed from simulated sequence reads, (4) meta-analysis using Stouffer's method across ancestries using hard genotypes, and (5) PCAngsd (MEISNER AND ALBRECHTSEN 2019) – were tested for variants deviating from HWE with various parameter settings, for low coverage (A-D) and high coverage (E-H) data. (A, E) Theta controls the degree of deviation from HWE, with negative values indicating excess heterozygosity and positive values indicating heterozygote depletion. The high Type I Error rates in GT-based tests (Figure 2) lead to those methods appearing to have higher power in some scenarios. The unadjusted test suffers from this problem the most. GL-based methods have slightly lower powers than GT-based methods in exchange for a much better controlled Type I error rate. This pattern mostly holds across different numbers of ancestries (B, F), p-value thresholds (C, G), and fixation indices (D, H). Meta-analysis had the lowest power in the presence of excess heterozygosity.

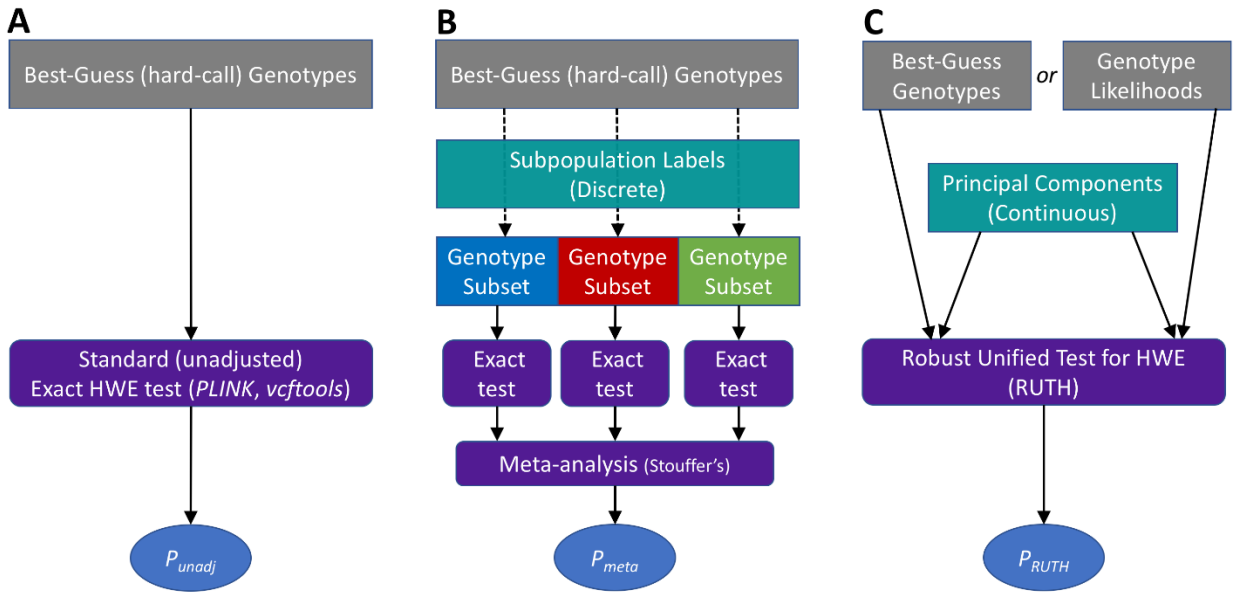

Figure 3

Schematic diagrams of different methods to test HWE under population structure. Three different methods to test HWE under population structure are described. (A) In the standard (unadjusted) HWE test, all samples are tested together using best-guess genotypes. This test does not adjust for sample ancestry. (B) In a meta-analysis of stratified HWE tests, the samples must first be categorized into discrete subpopulations, determined a priori based on their genotypes or self-reported ancestries. Next, standard HWE tests (based on best-guess genotypes) are performed on each of these subpopulations. Then, the resulting HWE statistics are converted into Z-scores and combined in a meta-analysis using Stouffer's method, with the sample sizes of the subpopulations as weights. (C) In our proposed method (RUTH), either best-guess genotypes or genotype likelihoods can be used as input for HWE test. We assume that the genetic ancestries of each sample are estimated a priori, typically as principal components (PCs). We combine the genotypes and PCs to perform either a score test or a likelihood ratio test to obtain a joint ancestry-adjusted HWE statistic for each variant across all samples.

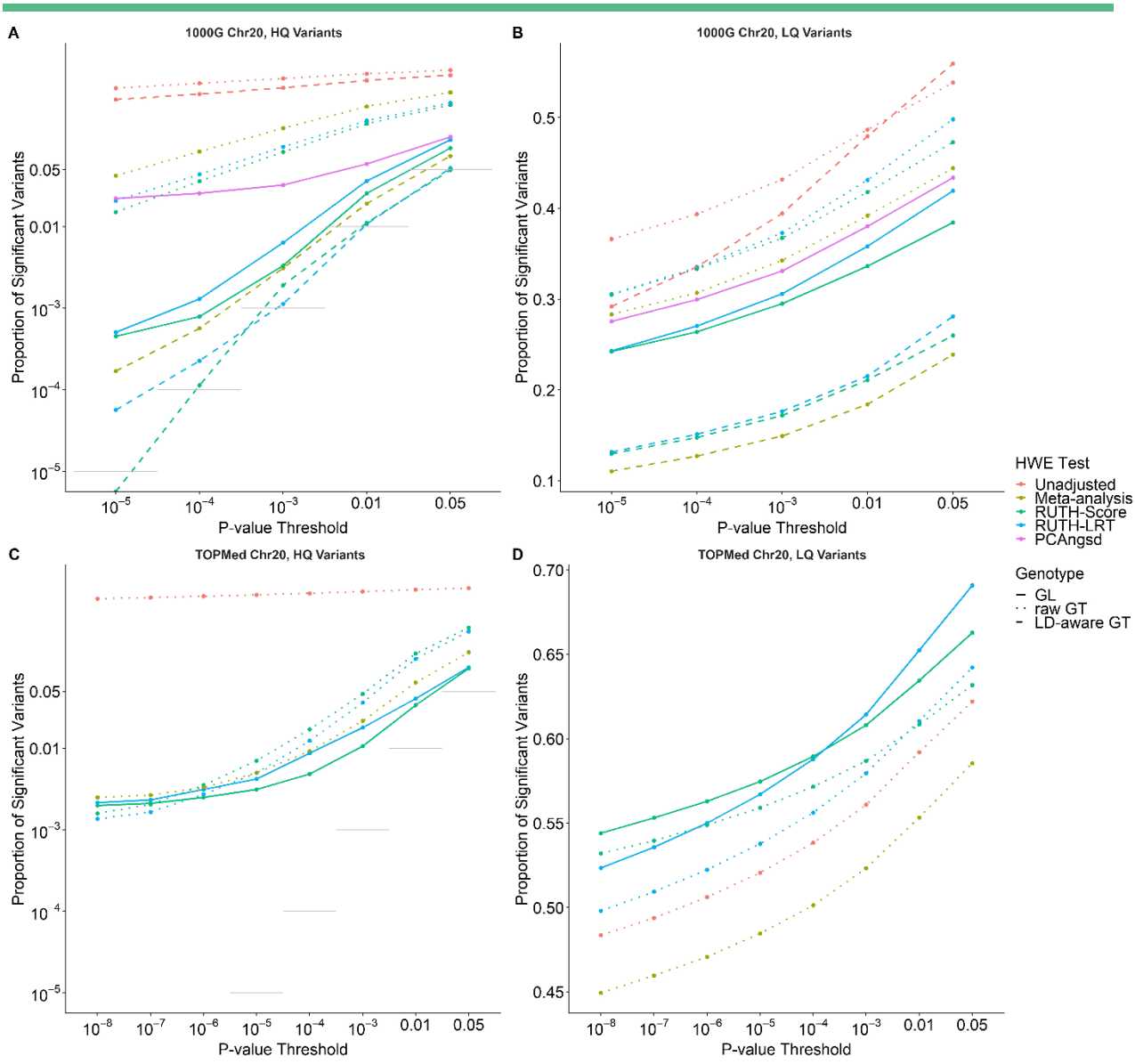

Figure 4

Evaluation of different HWE tests on 1000 Genomes and TOPMed variants. In 1000 Genomes data (A, B), we identified 17,740 "high quality" (HQ) variants and 10,966 "low quality" (LQ) variants in chromosome 20. In TOPMed data (C, D), we identified 17,524 HQ variants and 329,699 LQ variants in chromosome 20. A well-behaved HWE test should maximize the proportion of significant LQ variants while controlling the false positive rate for HQ variants. Dotted gray lines represent targeted Type I error levels if we assume all HQ variants follow HWE. (A) Both the unadjusted test and PCAngsd found substantially more significant variants than expected in the 1000G HQ variant set, while both RUTH and meta-analysis were more conservative. Methods that used raw GTs showed substantial false positive rates, while methods that used GLs and LD-aware GTs had much better control of false positives. (B) In 1000G LQ variants, meta-analysis lagged behind RUTH and the unadjusted test in discovering significant deviation from HWE. RUTH behaved well for HQ variants while having more power to find low-quality variants significantly deviating from HWE. (C) In TOPMed data, the unadjusted test resulted in an excess of false positives. Tests using GL-based genotypes outperformed tests using GT-based genotypes. (D) Methods using GL-based genotypes were able to discover more LQ variants than methods using GT-based genotypes, demonstrating the advantage of accounting for genotype uncertainty in HWE tests.

827 **Table 1**

828 Performance of the unadjusted test, meta-analysis, RUTH, and PCAngsd on 1000 Genomes chromosome 20
829 variants.

Variant Category	Genotype Format	HWE Test	Proportion of Significant Variants					Total Variant Count
			$P < 10^{-2}$	$P < 10^{-3}$	$P < 10^{-4}$	$P < 10^{-5}$	$P < 10^{-6}$	
LQ Variants	raw GT	Unadjusted	0.487	0.432	0.394	0.366	0.339	10,966
		Meta-analysis	0.392	0.343	0.307	0.283	0.262	10,966
		RUTH-Score	0.418	0.367	0.333	0.305	0.284	10,966
		RUTH-LRT	0.431	0.373	0.335	0.305	0.280	10,966
	LD-aware GT	Unadjusted	0.479	0.395	0.336	0.292	0.259	10,966
		Meta-analysis	0.184	0.149	0.127	0.111	0.098	10,966
		RUTH-Score	0.211	0.172	0.147	0.130	0.112	10,966
		RUTH-LRT	0.215	0.177	0.151	0.131	0.115	10,966
	GL	RUTH-Score	0.336	0.295	0.264	0.242	0.223	10,966
		RUTH-LRT	0.358	0.306	0.270	0.243	0.225	10,966
		PCAngsd	0.380	0.331	0.300	0.275	0.255	10,920
HQ Variants	raw GT	Unadjusted	0.755	0.657	0.573	0.501	0.443	17,740
		Meta-analysis	0.298	0.161	0.084	0.042	0.020	17,740
		RUTH-Score	0.183	0.083	0.036	0.015	7.4×10^{-3}	17,740
		RUTH-LRT	0.200	0.095	0.044	0.021	0.010	17,740
	LD-aware GT	Unadjusted	0.623	0.507	0.422	0.361	0.311	17,740
		Meta-analysis	0.019	3.1×10^{-3}	5.6×10^{-4}	1.7×10^{-4}	1.1×10^{-4}	17,740
		RUTH-Score	0.011	1.9×10^{-3}	1.1×10^{-4}	0	0	17,740
		RUTH-LRT	0.011	1.1×10^{-3}	2.3×10^{-4}	5.6×10^{-5}	0	17,740
	GL	RUTH-Score	0.026	3.3×10^{-3}	7.9×10^{-4}	4.5×10^{-4}	3.4×10^{-4}	17,740
		RUTH-LRT	0.036	6.4×10^{-3}	1.3×10^{-3}	5.1×10^{-4}	3.4×10^{-4}	17,740
		PCAngsd	0.059	0.032	0.026	0.022	0.021	17,740

830 The numbers within cells represent the proportions of significant variants under the corresponding testing
831 conditions at the given P-value threshold. We expect our LQ variants to violate HWE at a higher rate than our HQ
832 variants. A well-behaved test is expected to find a high proportion of LQ variants to be significant while
833 maintaining the targeted Type I Error rate in HQ variants. The unadjusted test consistently shows the highest false
834 positive rate among all the tests. HWE tests that rely on raw GTs also show much higher false positive rates than
835 tests that use other genotype representations. RUTH tests were the best at controlling false positives while still
836 maintaining comparable power to the other methods. PCAngsd had a much higher false positive rate than RUTH-
837 based methods, especially at more stringent p-value thresholds.
838

Table 2

Performance of the unadjusted test, meta-analysis, and RUTH on TOPMed freeze 5 chromosome 20 variants.

Variant set	Genotype Format	HWE Test	Proportion of Significant Variants					Total Variant Count
			$P < 10^{-2}$	$P < 10^{-3}$	$P < 10^{-4}$	$P < 10^{-5}$	$P < 10^{-6}$	
LQ Variants	raw GT	Unadjusted	0.592	0.561	0.539	0.521	0.506	329,699
	raw GT	Meta-analysis	0.554	0.524	0.502	0.485	0.471	329,699
	raw GT	RUTH-Score	0.608	0.587	0.572	0.559	0.549	329,699
	GL	RUTH-Score	0.635	0.608	0.590	0.575	0.563	329,699
	raw GT	RUTH-LRT	0.610	0.580	0.556	0.538	0.522	329,699
	GL	RUTH-LRT	0.653	0.615	0.588	0.567	0.550	329,699
HQ Variants	raw GT	Unadjusted	0.890	0.842	0.800	0.766	0.736	17,524
	raw GT	Meta-analysis	0.065	0.022	9.0×10^{-3}	4.8×10^{-3}	3.3×10^{-3}	17,524
	raw GT	RUTH-Score	0.145	0.047	0.172	7.1×10^{-3}	3.5×10^{-3}	17,524
	GL	RUTH-Score	0.034	0.011	4.9×10^{-3}	3.1×10^{-3}	2.5×10^{-3}	17,524
	raw GT	RUTH-LRT	0.125	0.036	0.012	5.0×10^{-3}	2.7×10^{-3}	17,524
	GL	RUTH-LRT	0.041	0.018	8.5×10^{-3}	4.3×10^{-3}	3.1×10^{-3}	17,524

The numbers within cells represent the proportions of significant variants under the corresponding testing conditions at the given P-value threshold. These results are based on tests that used likelihood-based genotype representations as input. A well-behaved test should reduce the number of significant high-quality (HQ) variants while increasing the number of significant low-quality (LQ) variants. The unadjusted test had a greatly inflated false positive rate for HQ variants while showing a lower true positive rate for LQ variants. While meta-analysis performed better for HQ variants, it had reduced power to find LQ variants to be significant. RUTH performed the best, with fewer false positives (significant HQ variants) compared to both the unadjusted test and meta-analysis, while at the same time finding more true positives (significant LQ variants).

852 **Table 3**

853 Runtimes for RUTH and PCAngsd on simulated data.

Sample Size	Wall Time (s)			User Time (s)		
	RUTH-LRT	RUTH-Score	PCAngsd	RUTH-LRT	RUTH-Score	PCAngsd
1,000	16.21	27.24	173.11	16.16	27.09	172.37
2,000	32.19	54.63	347.10	31.94	54.51	345.58
5,000	82.80	136.44	1,124.83	81.81	136.20	1,102.85
10,000	165.48	273.67	7,396.00	163.88	273.27	7,235.91
20,000	336.75	553.92	38,807.67	332.06	553.05	37,338.69
50,000	902.81	1,438.32	461,971.33	886.67	1,435.87	403,296.5

854

855 We simulated 10,000 genotype likelihood-based variants for varying numbers of samples. Wall time indicates total
856 runtime, while user time is the amount of time the CPUs spent running each program. All programs were run in
857 single-threaded mode. System processes make up the difference between the two values, with a majority
858 consisting of file I/O. We used VCF files with GL fields in RUTH and converted them to Beagle3 format for PCAngsd.
859 The RUTH likelihood ratio test (LRT) was the fastest method, with the score test about 60% slower. PCAngsd was
860 about 10 times slower than RUTH-LRT with the smallest sample sizes and over 400 times slower with our largest
861 tested size of 50,000 samples.
862

List of Supplementary Figures, Tables, and File

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Table S7. Sample contributions from each of the participating TOPMed studies.

Table S8. TOPMed acknowledgements for omics support.

File S1. TOPMed Study Acknowledgments

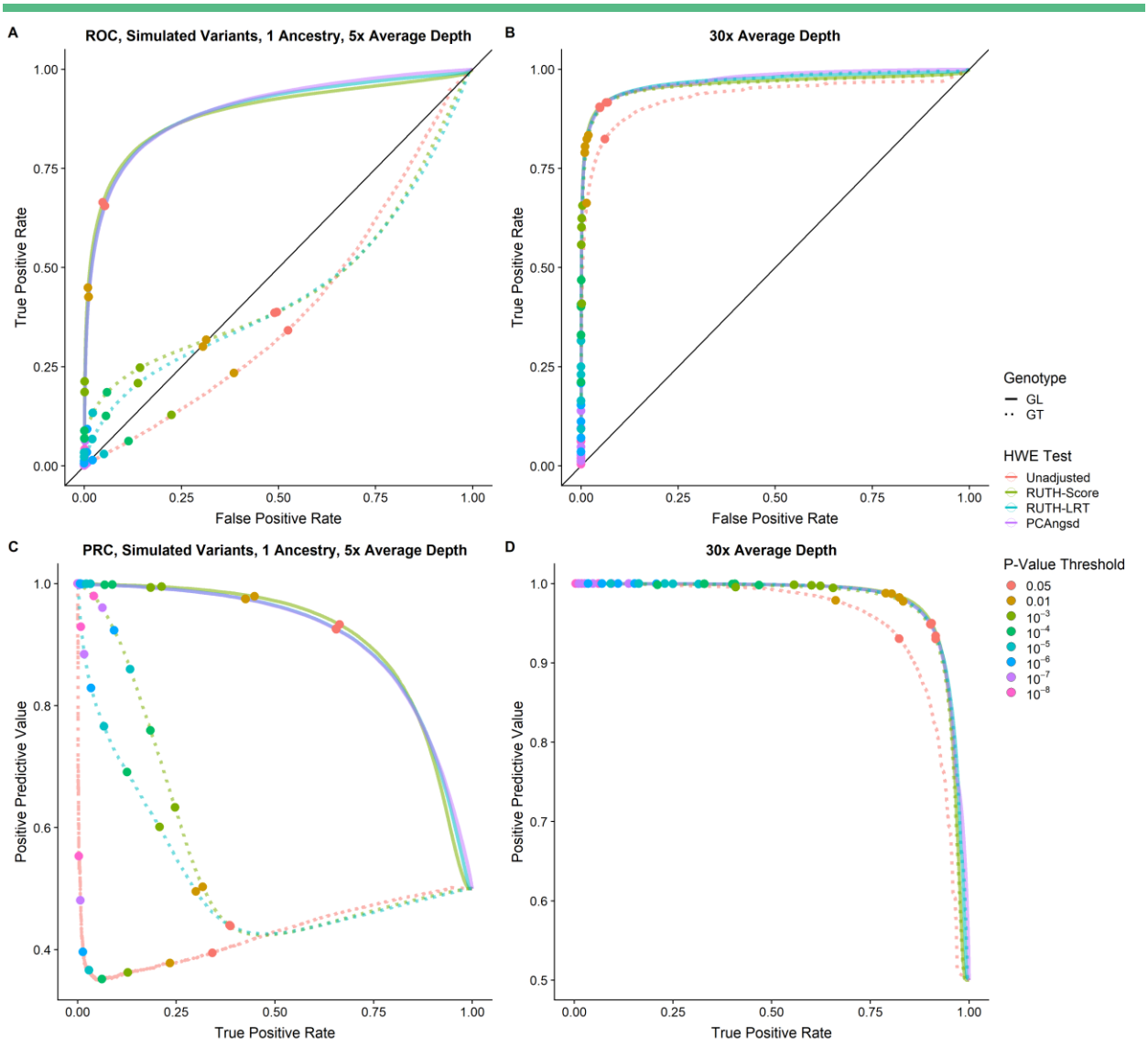

Figure S1

ROC and PRC for simulated single-ancestry data. For both low coverage (A, C) and high coverage (B, D) settings, 500,000 variants were generated from 5,000 samples arising from a single ancestry, with half of the variants as true positives ($\theta = -0.05$) and half of the variants as true negatives ($\theta = 0$). The colors of the lines correspond to the different HWE tests, while the colors of the points correspond to different P-value thresholds. In all cases, the unadjusted test performed the worst. For low-coverage data, tests using GT-based genotypes performed poorly due to their inability to capture the effects of genotype uncertainty, whereas tests using GL-based genotypes performed much better. The difference was negligible in high-coverage genotype data.

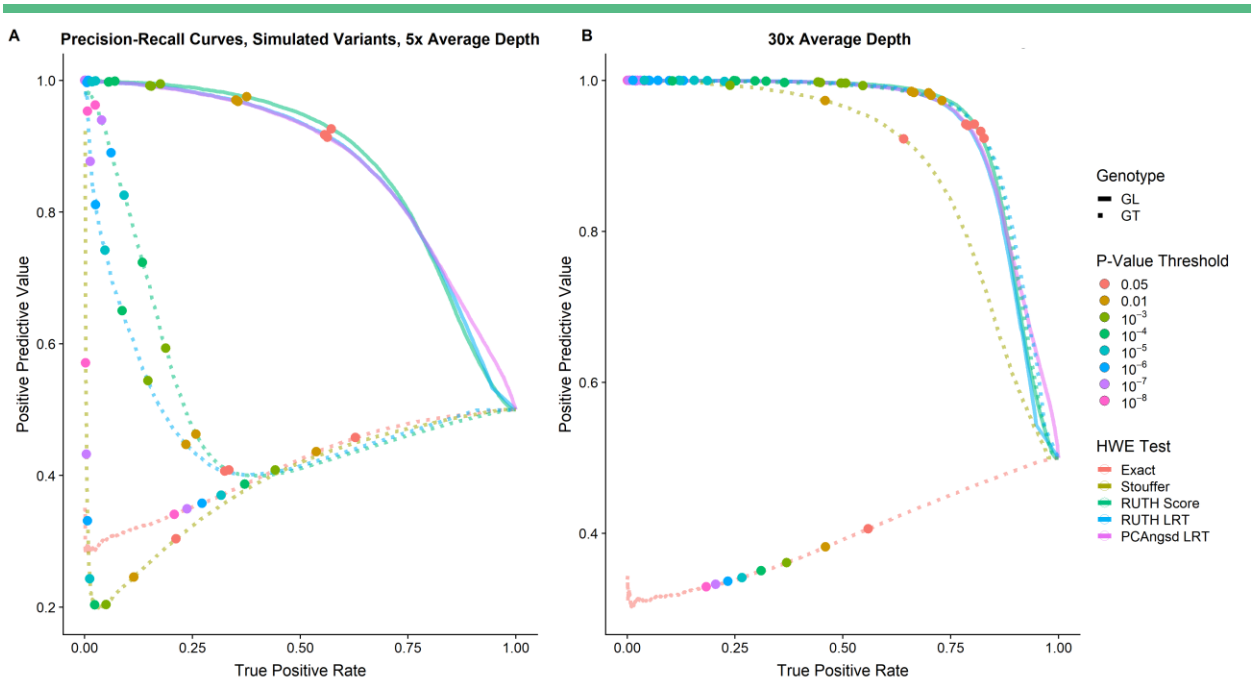

Figure S2

Precision-recall curves for simulated data with multiple ancestries. We generated Precision-recall curves to evaluate the tradeoff between the different HWE tests' ability to identify true positive variants while minimizing the misidentification of true negative variants as significantly departing from HWE. We analyzed 50,000 true positive and 50,000 true negative variants in 5,000 samples arising from 5 different ancestries with an average simulated depth of (A) 5x and (B) 30x. True negative variants are defined as variants with the HWE deviation parameter $\theta = 0$. True positives are defined as variants with $\theta = -0.05$. The True Positive Rate (TPR) is defined to be the proportion of variants with $\theta = -0.05$ that are significant at a given P-value threshold, while the Positive Predictive Value (PPV) is defined as the proportion of significant variants with $\theta = -0.05$ at the same P-value threshold. Selected p-value thresholds are indicated with colored circles. For low-depth genotypes, in the presence of high genotype uncertainty, GL-based HWE tests performed relatively well, while GT-based tests performed poorly. For high-depth genotypes, with low genotype uncertainty, all methods adjusting for population structure performed relatively well.

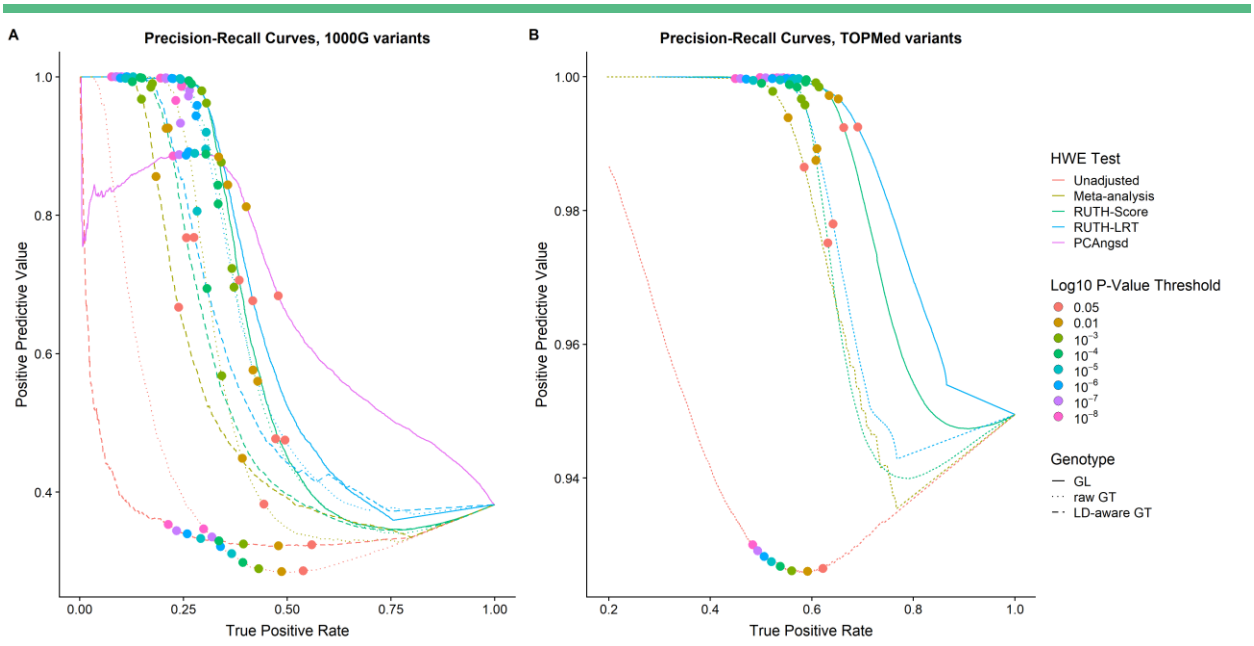

Figure S3

Precision-recall curves for 1000G and TOPMed variants. We defined positive variants as those with a high level of Mendelian inconsistency in family-based TOPMed data, and negative variants as those found in the intersection of the Illumina Omni2.5 and HapMap3 variant site lists. (A) For low-coverage sequence data found in 1000G, tests using GL-based genotypes (solid lines) generally performed better than tests using any GT-based genotypes (dotted and dashed lines). Both the unadjusted test and meta-analysis performed much worse than all other methods. (B) For high-coverage sequence data found in TOPMed, tests using GL-based genotypes retained their improved performance over tests using GT-based genotypes.

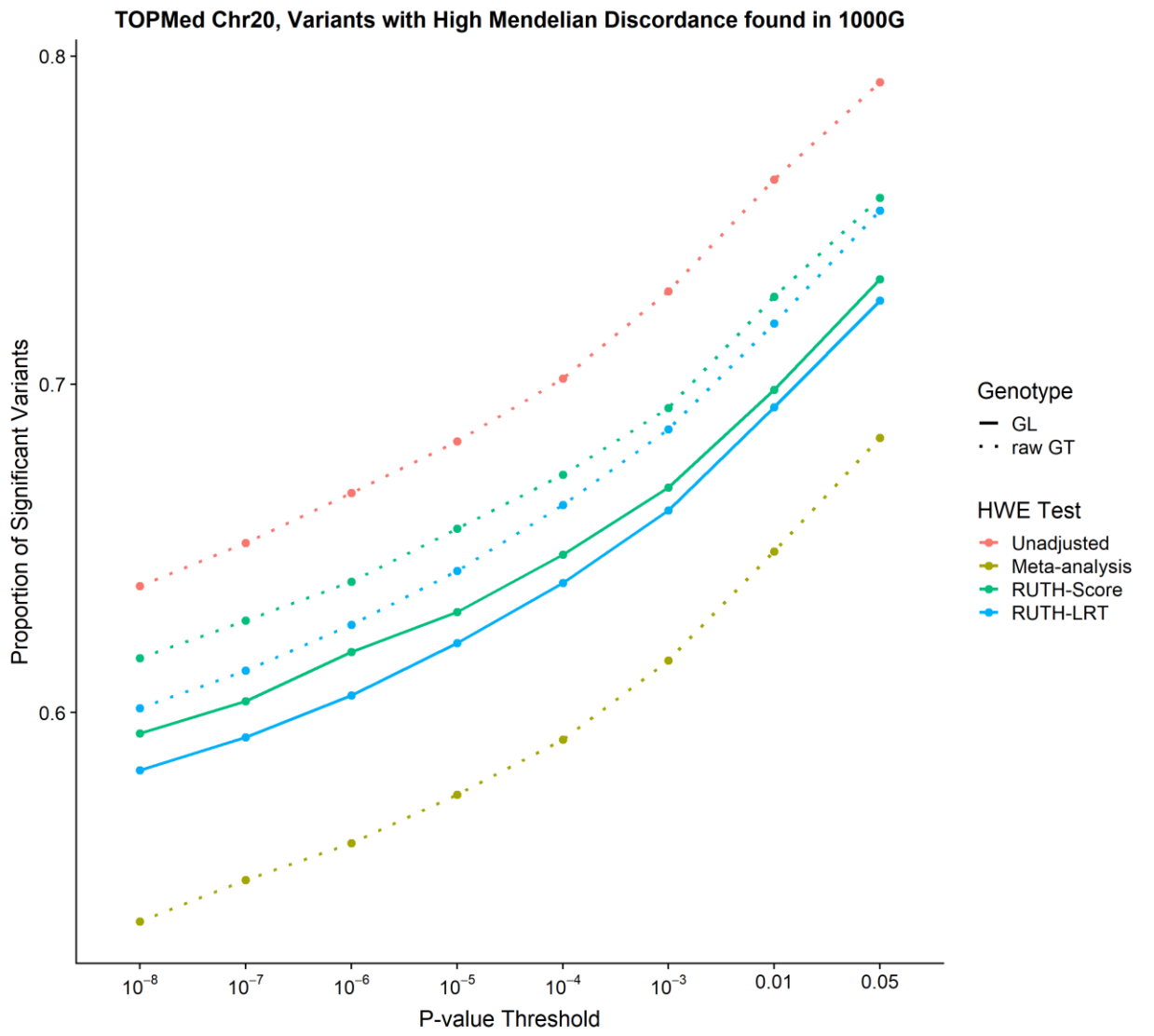

Figure S4

Results of testing TOPMed variants found in 1000G variant list. This analysis contains 10,966 TOPMed variants found to be discordant in TOPMed family data and overlapping with 1000G discordant variants, as opposed to all 329,699 discordant TOPMed variants (as seen in Figure 4D). Our results are similar to those for 1000G discordant variants (Figure 4B), suggesting that the differences between the patterns observed in 1000G and TOPMed results may have been caused by the difference in allele frequency distributions in the two data sets (Table S1).

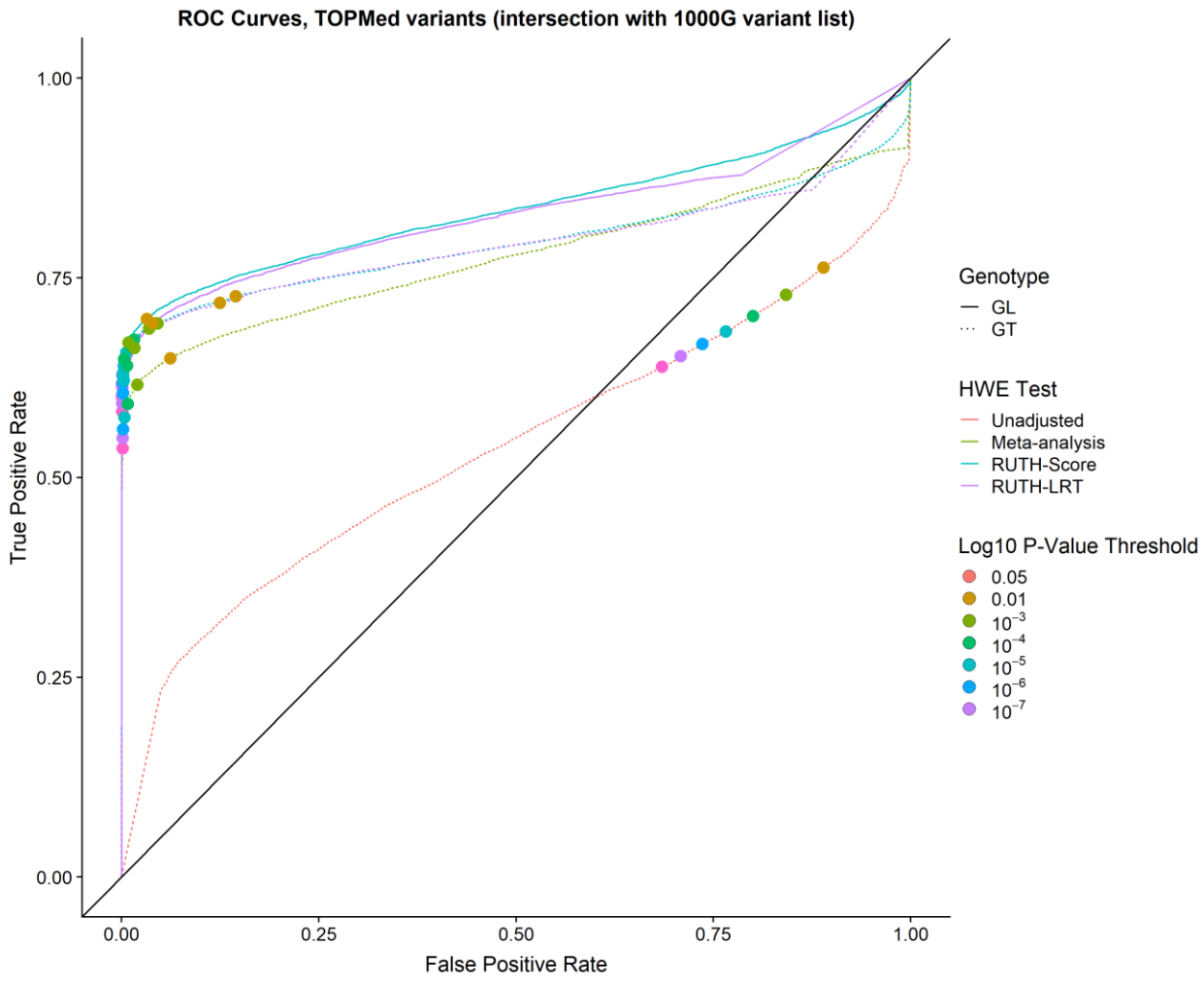

Figure S5

ROC curves for TOPMed variants found in 1000G variant list. GL-based tests have the best overall performance among the different methods.

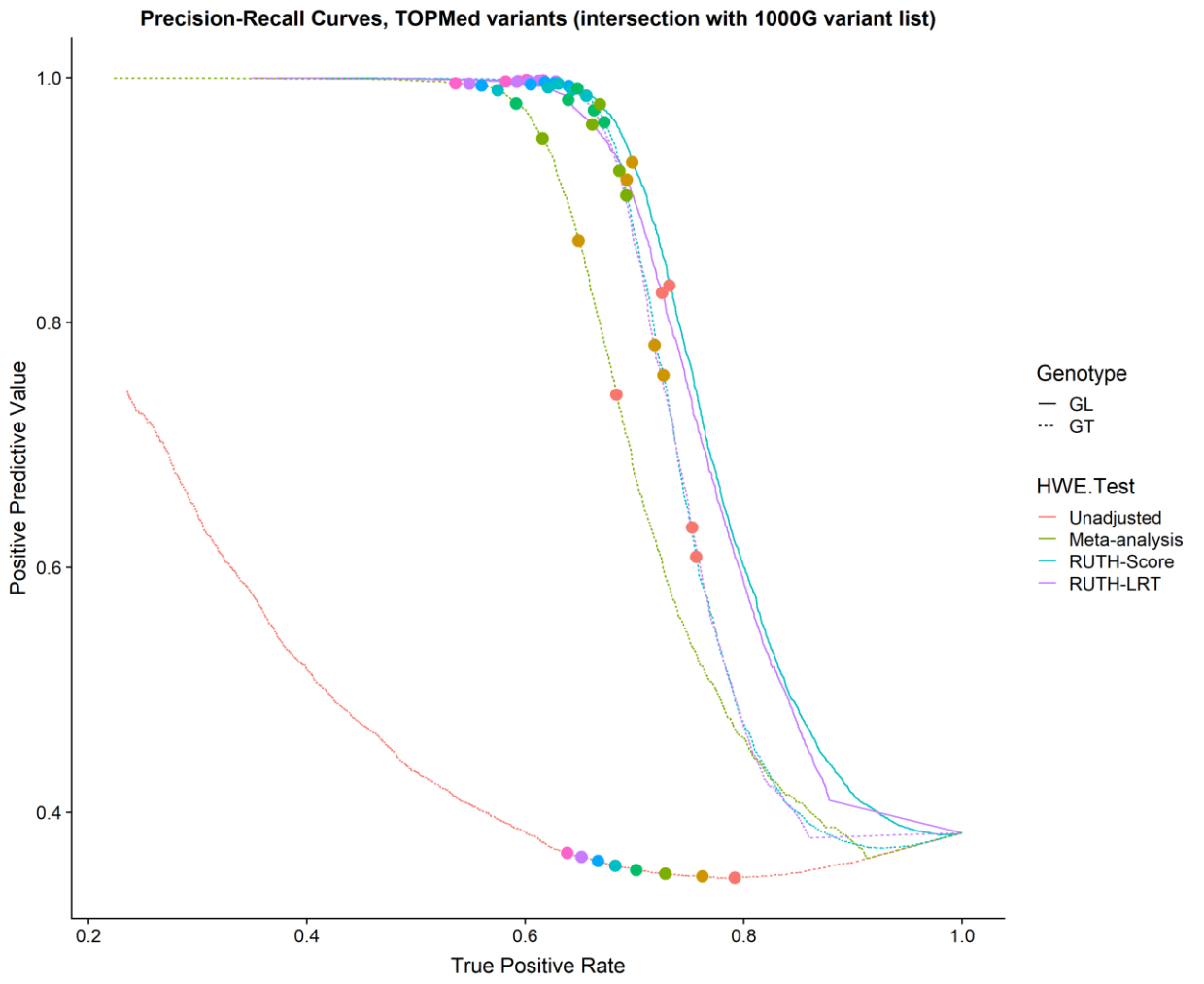

Figure S6

PRC curves for TOPMed variants found in 1000G variant list. RUTH tests using GLs offer the best balance between finding true positives and maximizing positive predictive value.

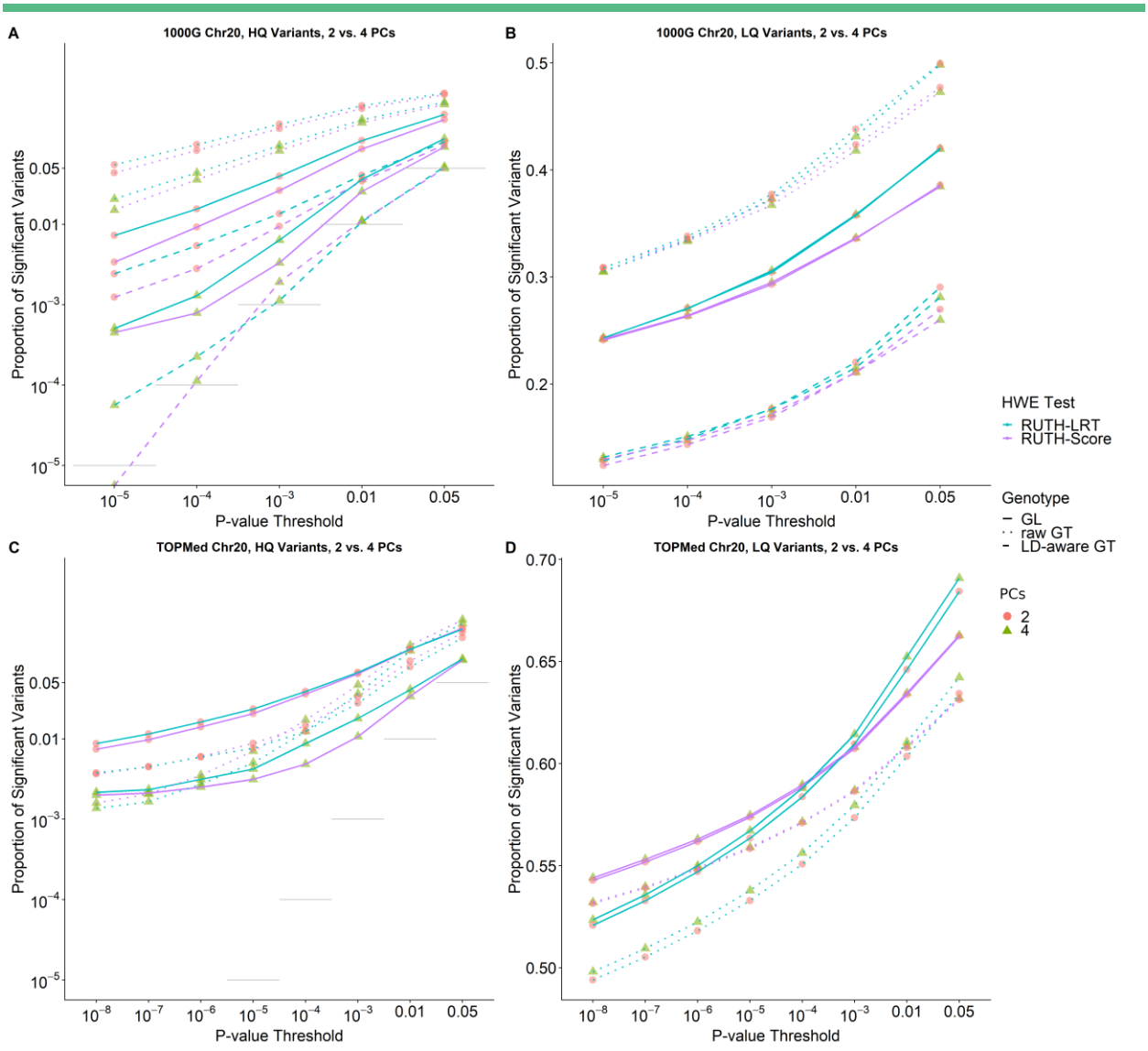

Figure S7

Results of testing 1000G and TOPMed variants with RUTH using two vs. four PCs. Using only 2 PCs lead to noticeably worse performance, especially for GL-based tests. (A) In 1000 Genomes data, using only 2 PCs leads to much higher false positives in HQ variants for both RUTH-Score and RUTH-LRT compared to using 4 PCs. (B) Tests on LQ variants with 2 PCs appear to have modestly higher power than tests using 4 PCs, but this is mainly due to the much higher false positive rate. (C) For HQ variants in TOPMed, tests using only 2 PCs have substantially higher false positive rate than tests using 4 PCs for GL-based tests, while GT-based tests are comparable. (D) Surprisingly, GL-based tests using 4 PCs discovered more significant LQ variants compared to GL-based tests using 2 PCs, even though GL-based tests using 2 PCs had a higher false positive rate in HQ variants.

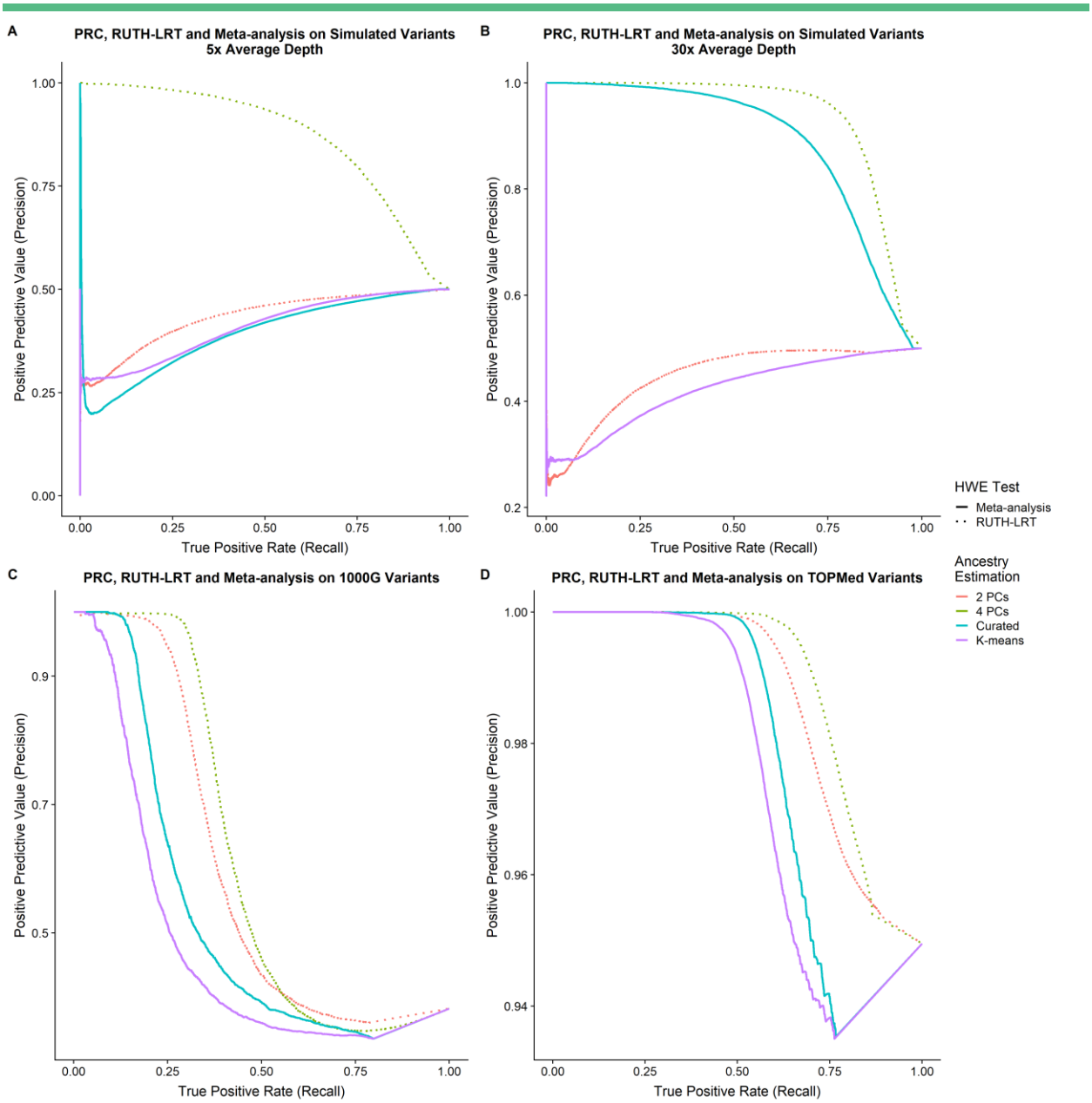

Figure S8

Effect of ancestry estimation accuracy on Precision-Recall Curves. We evaluated the effect of using 2 vs. 4 principal components on the performance of RUTH-LRT, and the effect of using our nearest-neighbor algorithm ("curated") vs. k-means for subpopulation classification of samples on the performance of meta-analysis on (A) low-depth simulated data, (B) high-depth simulated data, (C) 1000G variants, and (D) TOPMed variants. We simulated null variants with $\theta = 0$ and alternative variants with $\theta = -0.05$, with a fixation index of 0.1 for 5,000 samples from 5 ancestries (1,000 samples each). RUTH-LRT used GL-based genotypes, and meta-analysis used raw GT-based genotypes. K-means classification for simulated data was performed assuming 3 subpopulation clusters.

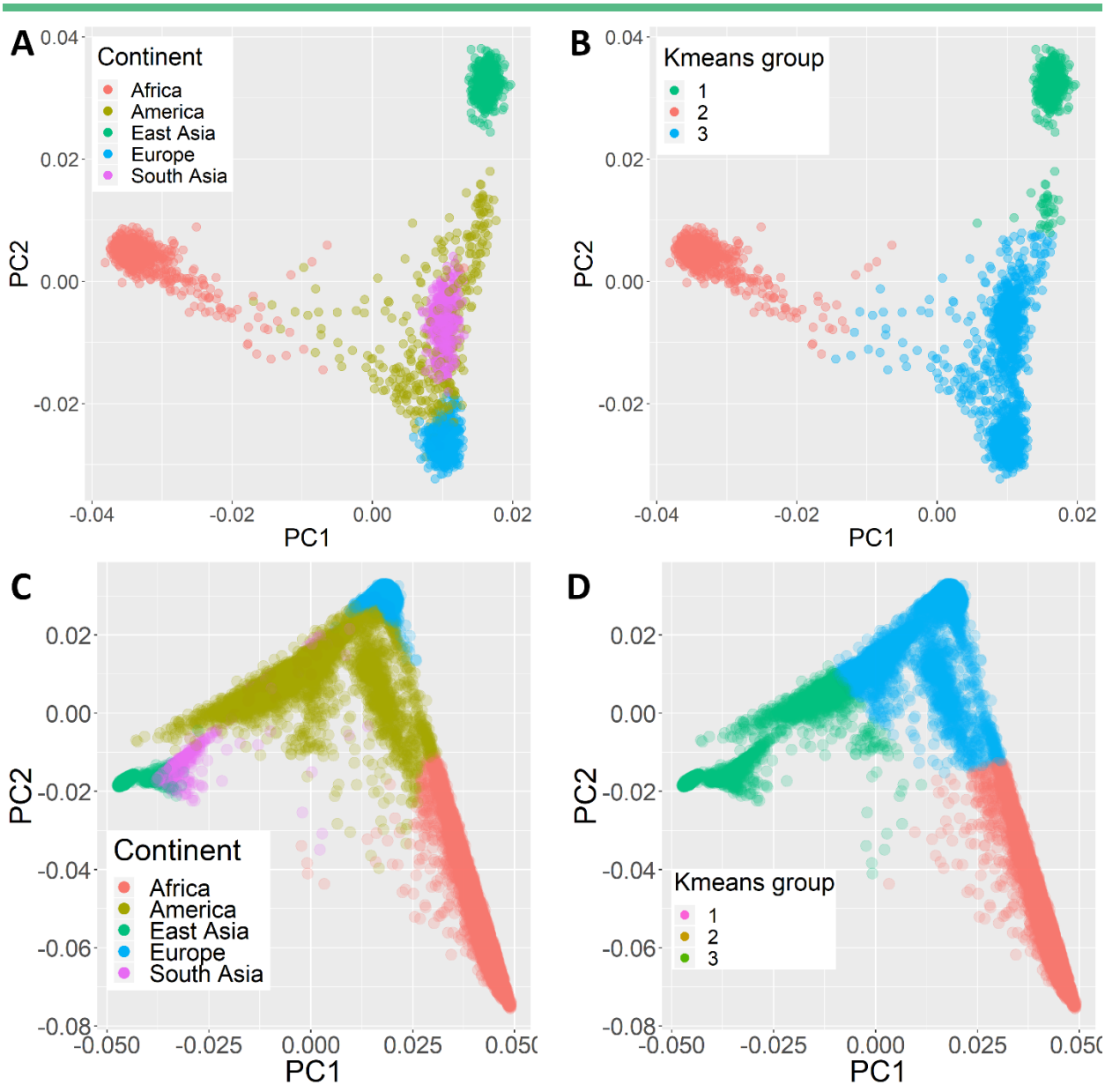

Figure S9

Principal component plots and group assignments for 1000 Genomes and TOPMed samples. Ancestry group assignments for samples in 1000G (A, B) and TOPMed (C, D) samples used either a high-quality ancestry estimation method (A, C) or a crude k-means based method (B, D). In meta-analysis, samples within a group were first analyzed together using the unadjusted test. Then, the group-level results were combined using Stouffer's method. Meta-analyses using the cruder k-means groupings performed much worse than those using the high-quality ancestry estimates due to population stratification within the cruder groups.

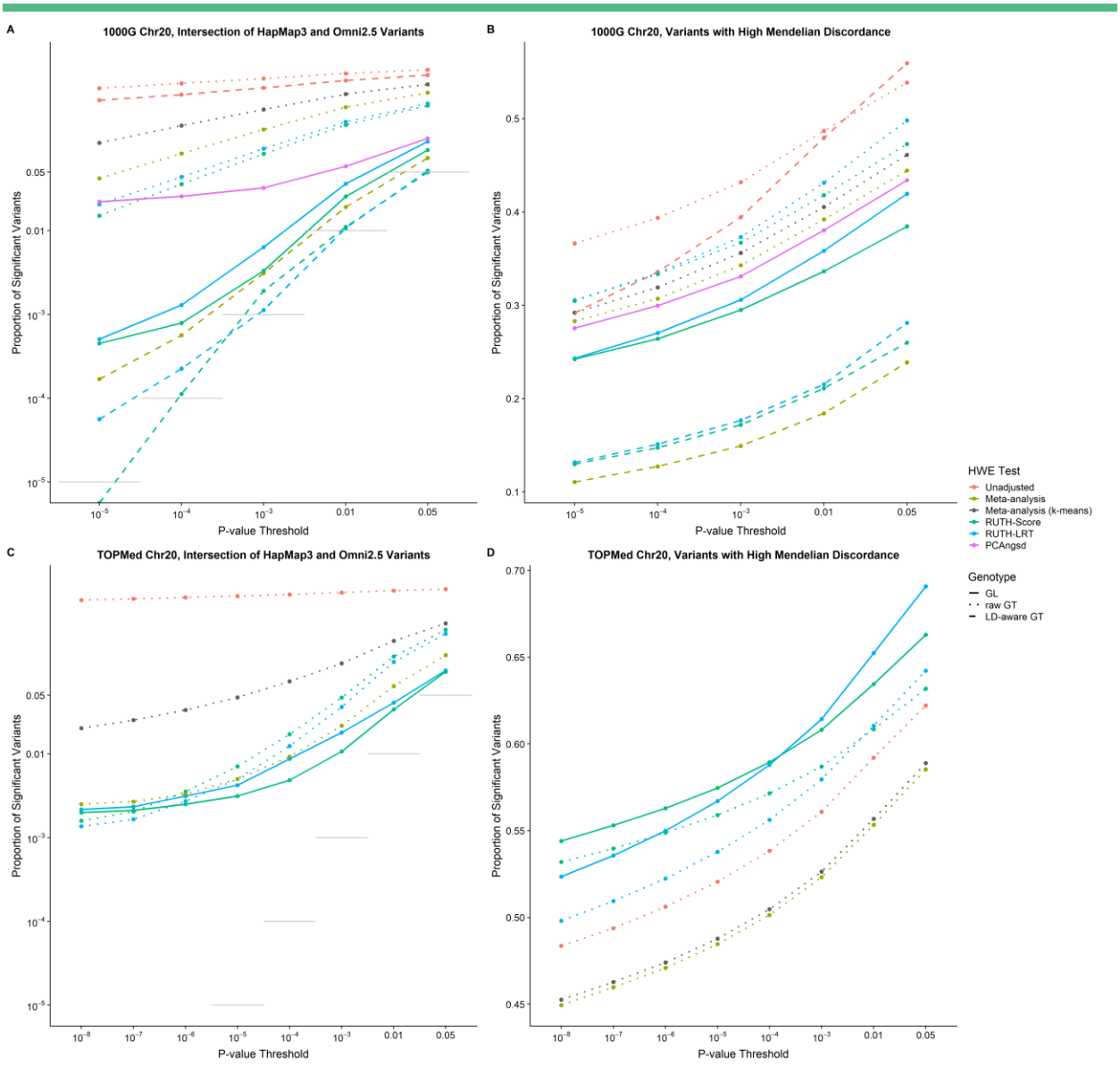

Figure S10

Results of testing 1000G and TOPMed variants with meta-analysis using K-means to generate ancestry groups. We generated three subpopulations for 1000G and TOPMed separately by applying k-means to the first two principal components of each group. Next, we calculated subpopulation-specific HWE statistics, which were converted to Z-scores and combined using Stouffer's method, using each subpopulation's size as the weights. (A) K-means-based meta-analysis had much higher false positive rates in 1000G compared to meta-analysis that used more accurate population labels, which (B) confounds its seemingly higher power to discover true positives. (C) We see the same increased false positive rate in K-means-based meta-analysis in TOPMed, but surprisingly (D) it also reduced the power to discover true positives in TOPMed. High-quality ancestry groups can substantially improve the performance of ancestry-based meta-analysis.

Table S1

Simulation results for the unadjusted test, meta-analysis, RUTH, and PCAngsd for HWE.

This table can be found at the following link:
https://docs.google.com/spreadsheets/d/1zdn7jOWgOMG_wwqwgDD4b1i0a2clGlyNFKmI5xR_DoE/edit?usp=sharing

Results from various HWE tests for simulations with 50,000 variants for 5,000 samples. Samples were generated using a population fixation index (F_{ST}) between .01 and .1. "GL" indicates a method using genotype likelihoods, while "GT" indicates a method using best-guess genotypes. Theta denotes deviation from HWE: Theta = 0 indicates no deviation from HWE, Theta < 0 indicates excess heterozygosity, and Theta > 0 indicates heterozygote depletion. When the samples were generated from a single ancestry, meta-analysis and the unadjusted test were identical. *Combined F_{ST} indicates the combined results for F_{ST}=.01, .02, .03, .05, and .1. This is available only when the number of ancestries is 1, because F_{ST} should not affect the results with single ancestry, so the results may be combined.

998 **Table S2**

999 Results from using lower quality ancestry estimations on meta-analysis and RUTH.

Data set	Variant set	Genotype Format	HWE Test	PCs	Proportion of Significant Variants					Total Variant Count
					P < 0.01	P < 10^{-3}	P < 10^{-4}	P < 10^{-5}	P < 10^{-6}	
1000G	LQ	raw GT	Meta-analysis	n/a	0.392	0.343	0.307	0.283	0.262	10,966
			Meta-analysis (k-means)	n/a	0.405	0.356	0.319	0.292	0.269	10,966
		LD-aware GT	Meta-analysis	n/a	0.184	0.149	0.127	0.111	0.098	10,966
			Meta-analysis (k-means)	n/a	0.221	0.169	0.136	0.116	0.102	10,966
	HQ	raw GT	Meta-analysis	n/a	0.298	0.161	0.084	0.042	0.020	17,740
			Meta-analysis (k-means)	n/a	0.427	0.279	0.180	0.112	0.067	17,740
		LD-aware GT	Meta-analysis	n/a	0.019	3.1x10^{-3}	5.6x10^{-4}	1.7x10^{-4}	1.1x10^{-4}	17,740
			Meta-analysis (k-means)	n/a	0.107	0.043	0.020	9.5x10^{-3}	5.0x10^{-3}	17,740
TOPMed	LQ	GT	Meta-analysis	n/a	0.553	0.523	0.501	0.485	0.471	329,699
			Meta-analysis (k-means)	n/a	0.557	0.526	0.505	0.488	0.474	329,699
	HQ		Meta-analysis	n/a	0.064	0.022	9.2x10^{-3}	5.0x10^{-3}	3.3x10^{-3}	17,524
			Meta-analysis (k-means)	n/a	0.224	0.121	0.074	0.047	0.033	17,524
1000G	LQ	GL	RUTH-LRT	2	0.357	0.304	0.271	0.243	0.224	10,966
				4	0.358	0.306	0.270	0.243	0.225	10,966
			RUTH-Score	2	0.336	0.293	0.263	0.241	0.221	10,966
				4	0.336	0.295	0.264	0.242	0.223	10,966
		LD-aware GT	RUTH-LRT	2	0.220	0.177	0.149	0.128	0.113	10,966
				4	0.215	0.177	0.151	0.131	0.115	10,966
			RUTH-Score	2	0.211	0.169	0.143	0.124	0.109	10,966
				4	0.211	0.172	0.147	0.130	0.112	10,966
		raw GT	RUTH-LRT	2	0.438	0.377	0.338	0.308	0.284	10,966
				4	0.431	0.373	0.335	0.305	0.28	10,966
			RUTH-Score	2	0.424	0.372	0.335	0.309	0.286	10,966
				4	0.418	0.367	0.333	0.305	0.284	10,966
	HQ	GL	RUTH-LRT	2	0.110	0.040	0.016	7.3x10^{-3}	3.3x10^{-3}	17,740
				4	0.036	6.4x10^{-3}	1.3x10^{-3}	5.1x10^{-4}	3.4x10^{-4}	17,740
			RUTH-Score	2	0.087	0.026	9.2x10^{-3}	3.4x10^{-3}	1.6x10^{-3}	17,740
				4	0.026	3.3x10^{-3}	7.9x10^{-4}	4.5x10^{-4}	3.4x10^{-4}	17,740
		LD-aware GT	RUTH-LRT	2	0.041	0.014	5.4x10^{-3}	2.4x10^{-3}	1.4x10^{-3}	17,740
				4	0.011	1.1x10^{-3}	2.3x10^{-4}	5.6x10^{-5}	0	17,740
			RUTH-Score	2	0.034	9.5x10^{-3}	2.8x10^{-3}	1.2x10^{-3}	5.1x10^{-4}	17,740
				4	0.011	1.9x10^{-3}	1.1x10^{-4}	0	0	17,740
		raw GT	RUTH-LRT	2	0.299	0.176	0.098	0.055	0.03	17,740
				4	0.200	0.095	0.044	0.021	9.7x10^{-3}	17,740
			RUTH-Score	2	0.276	0.155	0.083	0.044	0.023	17,740
				4	0.183	0.083	0.036	0.015	7.4x10^{-3}	17,740
TOPMed	LQ	GL	RUTH-LRT	2	0.646	0.610	0.584	0.563	0.547	329,699
				4	0.652	0.614	0.588	0.567	0.55	329,699
			RUTH-Score	2	0.634	0.607	0.589	0.574	0.562	329,699
				4	0.635	0.608	0.590	0.575	0.562	329,699
		GT	RUTH-LRT	2	0.603	0.573	0.551	0.533	0.518	329,699
				4	0.610	0.580	0.556	0.538	0.552	329,699
			RUTH-Score	2	0.608	0.586	0.571	0.558	0.548	329,699
				4	0.608	0.587	0.572	0.559	0.549	329,699
	HQ	GL	RUTH-LRT	2	0.130	0.067	0.039	0.024	0.016	17,524
				4	0.041	0.018	8.7x10^{-3}	4.2x10^{-3}	3.1x10^{-3}	17,524
			RUTH-Score	2	0.130	0.065	0.036	0.021	0.014	17,524
				4	0.034	0.011	4.9x10^{-3}	3.1x10^{-3}	2.5x10^{-3}	17,524
		GT	RUTH-LRT	2	0.079	0.028	0.012	7.6x10^{-3}	5.9x10^{-3}	17,524
				4	0.125	0.036	0.012	5.0x10^{-3}	2.7x10^{-3}	17,524
			RUTH-Score	2	0.093	0.033	0.015	8.8x10^{-3}	6.0x10^{-3}	17,524
				4	0.145	0.047	0.017	7.1x10^{-3}	3.5x10^{-3}	17,524

1000 In both 1000G and TOPMed, the false positive rate was much higher when k-means-based groupings were used for
1001 meta-analysis, compared to when high quality ancestry groupings were used. Similarly, the false positive rate was
1002 much higher when only 2 PCs were used, compared to when 4 PCs were used. Surprisingly, in TOPMed, using 4 PCs
1003 led to both a lower false positive rate and higher true positive rate when compared to using 2 PCs.

Table S3

Performance of the unadjusted test, meta-analysis, and RUTH on the subset of TOPMed freeze 5 chromosome 20 variants that are also found in 1000G.

Variant set	Genotype Format	HWE Test	Proportion of Significant Variants					Total Variant Count
			$P < 10^{-2}$	$P < 10^{-3}$	$P < 10^{-4}$	$P < 10^{-5}$	$P < 10^{-6}$	
HQ Variants	raw GT	Unadjusted	0.890	0.842	0.800	0.766	0.736	16,924
	raw GT	Meta-analysis	0.062	0.020	8.0×10^{-3}	3.8×10^{-3}	2.3×10^{-3}	16,924
	raw GT	RUTH-Score	0.145	0.046	0.016	6.3×10^{-3}	2.8×10^{-3}	16,924
	GL	RUTH-Score	0.032	9.3×10^{-3}	3.7×10^{-3}	2.0×10^{-3}	1.5×10^{-3}	16,924
	raw GT	RUTH-LRT	0.125	0.035	0.011	4.2×10^{-3}	1.9×10^{-3}	16,924
	GL	RUTH-LRT	0.039	0.016	7.4×10^{-3}	3.1×10^{-3}	2.2×10^{-3}	16,924
LQ Variants	raw GT	Unadjusted	0.762	0.728	0.702	0.683	0.667	10,513
	raw GT	Meta-analysis	0.649	0.616	0.592	0.575	0.560	10,513
	raw GT	RUTH-Score	0.727	0.693	0.673	0.656	0.640	10,513
	GL	RUTH-Score	0.698	0.669	0.648	0.631	0.618	10,513
	raw GT	RUTH-LRT	0.719	0.686	0.663	0.643	0.627	10,513
	GL	RUTH-LRT	0.693	0.662	0.639	0.621	0.605	10,513

For HQ variants, GL-based HWE tests had much better control of false positives than GT-based tests. Conversely, for LQ variants, GT-based HWE tests had a slightly better true positive rate than GL-based tests. Overall, GL-based tests had the best performance when considering the tradeoff between false positives and true positives (Figure S5-6).

Table S4

Simulation results for RUTH tests using 2 vs 4 principal components.

This table can be found at the following link:
https://docs.google.com/spreadsheets/d/1Ac9rveZax5Y8NlKQ47wBaJNELqeJkFuNUpa1sNgnsno/edit?usp=sharing
We tested the effect of using different numbers of PCs in RUTH on Type I Error ($\theta = 0$) and power ($\theta \neq 0$) for simulated samples with different numbers of ancestries, fixation indices, sequencing depths, and genotype representations. We simulated 50,000 variants for each combination of simulation parameters.

Table S5

The effect of high vs. low quality subpopulation classification on meta-analysis in simulated samples.

Grouping	Depth	Theta	Proportion of significant variants				
			$P < 10^{-6}$	$P < 10^{-5}$	$P < 10^{-4}$	$P < 10^{-3}$	$P < 0.01$
True ancestry labels	5	-0.05	0.0073	0.0125	0.0235	0.05	0.1145
		0	0.0147	0.0388	0.0919	0.1955	0.3519
	30	-0.05	0.0139	0.04	0.1048	0.2389	0.4594
		0	0	0	0.0001	0.0016	0.0127
k-means (3 groups)	5	-0.05	0.1201	0.149	0.19	0.2509	0.3513
		0	0.2907	0.3496	0.4195	0.4977	0.5826
	30	-0.05	0.0919	0.1122	0.1447	0.2017	0.3097
		0	0.2183	0.2553	0.3054	0.3734	0.4747

We simulated 50,000 variants in 5,000 samples arising from 5 distinct subpopulations (1,000 samples each), at low (5x) and high (30x) depth, with no deviation from HWE ($\theta = 0$) and moderate excess heterozygosity ($\theta = -0.05$). We used one of two different groupings for our samples: for high-quality labels, we used the original true ancestry labels from which we simulated our data; for low-quality labels, we ran k-means classification on the first 2 principal components of genetic variation for all our samples to generate 3 groups. We meta-analyzed all data sets using Stouffer's method. Type I error rates for low-depth samples were greatly inflated. For high-depth samples, when we used the true ancestry labels, Type I errors were well-controlled, with reasonable power to discover deviations from HWE, while when we used the crude k-means labels, Type I errors were greatly inflated, with surprisingly less power to discover deviations from HWE at less stringent P-value thresholds. These results highlight the importance of high-quality subpopulation classification for meta-analysis.

Table S6

Comparison of runtimes and memory requirements for RUTH and PCAngsd in simulated and 1000G data.

Data set	Genotype Format	Software	Test	N	Total Variant Count	Runtime (s)	Memory requirement (MB)
Simulated	GT	PLINK	Unadjusted	5,000	50,000	22	10
	GT	RUTH	RUTH LRT	5,000	50,000	348	15
	GL	RUTH	RUTH LRT	5,000	50,000	341	15
	GT	RUTH	RUTH Score	5,000	50,000	460	15
	GL	RUTH	RUTH Score	5,000	50,000	469	15
Simulated (5x)	GL	PCAngsd	PCAngsd	5,000	50,000	6,068	6,946
Simulated (30x)	GL	PCAngsd	PCAngsd	5,000	50,000	5,337	6,872
1000G	GT	PLINK	Unadjusted	2,504	28,706	2	8
	GL	RUTH	RUTH LRT	2,504	28,706	147	14
	GT	RUTH	RUTH LRT	2,504	28,706	96	13
	GL	RUTH	RUTH Score	2,504	28,706	216	14
	GT	RUTH	RUTH Score	2,504	28,706	177	13
	GL	PCAngsd	PCAngsd	2,504	28,660	4,105	2,073
TOPMed	GT	RUTH	RUTH LRT	53,831	347,223	158,731	57
	GL	RUTH	RUTH LRT	53,831	347,223	196,169	57

Simulation runtimes for PLINK and RUTH are averaged over 360 runs, across combinations of different simulation parameters. Simulation results for PCAngsd are averaged over 66 runs each for 5x and 30x coverage data. The higher uncertainty in low depth simulated data appears to have led to slower convergence in PCAngsd. All results for 1000G were from single runs. The listed TOPMed runtimes and memory requirements are for single-threaded analyses of all variants.

1044 **Table S7**

TOPMed Study Name	TOPMed Accession	Sample Size
Genetics of Cardiometabolic Health in the Amish	phs000956	1,025
Trans-Omics for Precision Medicine Whole Genome Sequencing Project: ARIC	phs001211	3,585
The Genetics and Epidemiology of Asthma in Barbados	phs001143	944
Cleveland Clinic Atrial Fibrillation Study	phs001189	328
The Cleveland Family Study (WGS)	phs000954	919
Cardiovascular Health Study	phs001368	69
Genetic Epidemiology of COPD (COPDGene) in theTOPMed Program	phs000951	8,733
The Genetic Epidemiology of Asthma in Costa Rica	phs000988	1,040
Diabetes Heart Study African American Coronary Artery Calcification (AA CAC)	phs001412	322
Whole Genome Sequencing and Related Phenotypes in the Framingham Heart Study	phs000974	3,725
Genes-environments and Admixture in Latino Asthmatics (GALA II) Study	phs000920	912
GeneSTAR (Genetic Study of Atherosclerosis Risk)	phs001218	1,633
Genetic Epidemiology Network of Arteriopathy (GENOA)	phs001345	1,069
Genetic Epidemiology Network of Salt Sensitivity (GenSalt)	phs001217	1,680
Genetics of Lipid Lowering Drugs and Diet Network (GOLDN)	phs001359	892
Heart and Vascular Health Study (HVH)	phs000993	64
HyperGEN - Genetics of Left Ventricular (LV) Hypertrophy	phs001293	1,752
Jackson Heart Study	phs000964	3,074
Whole Genome Sequencing of Venous Thromboembolism (WGS of VTE)	phs001402	1,250
MESA and MESA Family AA-CAC	phs001416	4,804
MGH Atrial Fibrillation Study	phs001062	916
Partners HealthCare Biobank	phs001024	109
San Antonio Family Heart Study (WGS)	phs001215	1,478
Study of African Americans, Asthma, Genes and Environment (SAGE) Study	phs000921	450
African American Sarcoidosis Genetics Resource	phs001207	606
Genome-wide Association Study of Adiposity in Samoans	phs000972	1,198
The Vanderbilt AF Ablation Registry	phs000997	154
The Vanderbilt Atrial Fibrillation Registry	phs001032	1016
Novel Risk Factors for the Development of Atrial Fibrillation in Women	phs001040	97
Women's Health Initiative (WHI)	phs001237	9,984
Total		53,831

1045 Sample contributions from each of the participating TOPMed studies.

1046

1047 **Table S8**

TOPMed Accession #	TOPMed Project	Parent Study	TOPMed Phase	Omics Center	Omics Support
phs000956	Amish	Amish	1	Broad Genomics	3R01HL121007-01S1
phs001211	AFGen	ARIC AFGen	1	Broad Genomics	3R01HL092577-06S1
phs001211	VTE	ARIC	2	Baylor	3U54HG003273-12S2 / HHSN268201500015C
phs001143	BAGS	BAGS	1	Illumina	3R01HL104608-04S1
phs001189	AFGen	CCAF	1	Broad Genomics	3R01HL092577-06S1
phs000954	CFS	CFS	1	NWGC	3R01HL098433-05S1
phs000954	CFS	CFS	3.5	NWGC	HHSN268201600032I
phs001368	CHS	CHS	3	Baylor	HHSN268201600033I
phs001368	VTE	CHS VTE	2	Baylor	3U54HG003273-12S2 / HHSN268201500015C
phs000951	COPD	COPDGene	1	NWGC	3R01HL089856-08S1
phs000951	COPD	COPDGene	2	Broad Genomics	HHSN268201500014C
phs000951	COPD	COPDGene	2.5	Broad Genomics	HHSN268201500014C
phs000988	CRA_CAMP	CRA	1	NWGC	3R37HL066289-13S1
phs000988	CRA_CAMP	CRA	3	NWGC	HHSN268201600032I
phs001412	AA_CAC	DHS	2	Broad Genomics	HHSN268201500014C
phs000974	AFGen	FHS AFGen	1	Broad Genomics	3R01HL092577-06S1
phs000974	FHS	FHS	1	Broad Genomics	3U54HG003067-12S2
phs000920	ATGC	GALAII ATGC	3	NWGC	HHSN268201600032I
phs000920	PGX_Asthma	GALAII	1	NYGC	3R01HL117004-02S3
phs001218	AA_CAC	GeneSTAR AA_CAC	2	Broad Genomics	HHSN268201500014C
phs001218	GeneSTAR	GeneSTAR	legacy	Illumina	R01HL112064
phs001218	GeneSTAR	GeneSTAR	2	Psomagen	3R01HL112064-04S1
phs001345	HyperGEN_GENOA	GENOA	2	NWGC	3R01HL055673-18S1
phs001345	AA_CAC	GENOA AA_CAC	2	Broad Genomics	HHSN268201500014C
phs001217	GenSalt	GenSalt	2	Baylor	HHSN268201500015C
phs001359	GOLDN	GOLDN	2	NWGC	3R01HL104135-04S1
phs000993	AFGen	HVH	1	Broad Genomics	3R01HL092577-06S1
phs000993	VTE	HVH VTE	2	Baylor	3U54HG003273-12S2 / HHSN268201500015C
phs001293	HyperGEN_GENOA	HyperGEN	2	NWGC	3R01HL055673-18S1
phs000964	JHS	JHS	1	NWGC	HHSN268201100037C
phs001402	VTE	Mayo_VTE	2	Baylor	3U54HG003273-12S2 / HHSN268201500015C
phs001416	AA_CAC	MESA AA_CAC	2	Broad Genomics	HHSN268201500014C
phs001416	MESA	MESA	2	Broad Genomics	3U54HG003067-13S1
phs001062	AFGen	MGH_AF	1.4; 1.5; 2.4	Broad Genomics	3U54HG003067-12S2 / 3U54HG003067-13S1; 3U54HG003067-12S2 / 3U54HG003067-13S1; 3UM1HG008895-01S2
phs001062	AFGen	MGH_AF	1	Broad Genomics	3R01HL092577-06S1
phs001024	AFGen	Partners	1	Broad Genomics	3R01HL092577-06S1
phs001215	SAFS	SAFS	1	Illumina	3R01HL113323-03S1
phs001215	SAFS	SAFS	legacy	Illumina	R01HL113322
phs000921	ATGC	SAGE ATGC	3	NWGC	HHSN268201600032I
phs000921	PGX_Asthma	SAGE	1	NYGC	3R01HL117004-02S3
phs000972	Samoan	Samoan	1	NWGC	HHSN268201100037C
phs000972	Samoan	Samoan	2	NYGC	HHSN268201500016C
phs001207	Sarcoidosis	Sarcoidosis	2	Baylor	3R01HL113326-04S1
phs001207	Sarcoidosis	Sarcoidosis	3.5	NWGC	HHSN268201600032I
phs000997	AFGen	VAFAR	1.5; 2.4; 5.3	Broad Genomics	3U54HG003067-12S2 / 3U54HG003067-13S1; 3UM1HG008895-01S2; 3UM1HG008895-01S2

phs000997	AFGen	VAFAR	1	Broad Genomics	3R01HL092577-06S1
phs001032	AFGen	VU_AF	1	Broad Genomics	3R01HL092577-06S1
phs001040	AFGen	WGHS	1	Broad Genomics	3R01HL092577-06S1
phs001237	WHI	WHI	2	Broad Genomics	HHSN268201500014C

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File S1

TOPMed Study Acknowledgements

NHLBI TOPMed: Genetics of Cardiometabolic Health in the Amish

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NHLBI TOPMed: Trans-Omics for Precision Medicine Whole Genome Sequencing Project: ARIC

Genome Sequencing for “NHLBI TOPMed: Atherosclerosis Risk in Communities (ARIC)” (phs001211) was performed at the Baylor College of Medicine Human Genome Sequencing Center (HHSN268201500015C and 3U54HG003273-12S2) and the Broad Institute of MIT and Harvard (3R01HL092577-06S1).

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NHLBI TOPMed: The Genetics and Epidemiology of Asthma in Barbados

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NHLBI TOPMed: Cleveland Clinic Atrial Fibrillation Study

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NHLBI TOPMed: The Cleveland Family Study (WGS)

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NHLBI TOPMed: Cardiovascular Health Study

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NHLBI TOPMed: Genetic Epidemiology of COPD (COPDGene) in the TOPMed Program

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NHLBI TOPMed: The Genetic Epidemiology of Asthma in Costa Rica

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NHLBI TOPMed: Diabetes Heart Study African American Coronary Artery Calcification (AA CAC)

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NHLBI TOPMed: Whole Genome Sequencing and Related Phenotypes in the Framingham Heart Study

The Framingham Heart Study (FHS) is a prospective cohort study of 3 generations of subjects who have been followed up to 65 years to evaluate risk factors for cardiovascular disease.13-16 Its large sample of ~15,000 men and women who have been extensively phenotyped with repeated examinations make it ideal for the study of genetic associations with cardiovascular disease risk factors and outcomes. DNA samples have been collected and immortalized since the mid-1990s and are available on ~8000 study participants in 1037 families. These samples have been used for collection of GWAS array data and exome chip data in nearly all with DNA samples, and for targeted sequencing, deep exome sequencing and light coverage whole genome sequencing in limited numbers. Additionally, mRNA and miRNA expression data, DNA methylation data, metabolomics and other 'omics data are available on a sizable portion of study participants. This project will focus on deep whole genome sequencing (mean 30X coverage) in ~4100 subjects and imputed to all with GWAS array data to more fully understand the genetic contributions to cardiovascular, lung, blood and sleep disorders.

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NHLBI TOPMed: Genes-environments and Admixture in Latino Asthmatics (GALA II) Study

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The GALA II study collaborators include Shannon Thyne, UCSF; Harold J. Farber, Texas Children's Hospital; Denise Serebrisky, Jacobi Medical Center; Rajesh Kumar, Lurie Children's Hospital of Chicago; Emerita Brigino-Buenaventura, Kaiser Permanente; Michael A. LeNoir, Bay Area Pediatrics; Kelley Meade, UCSF Benioff Children’s Hospital, Oakland; William Rodriguez-Cintron, VA Hospital, Puerto Rico; Pedro C. Avila, Northwestern University; Jose R. Rodriguez-Santana, Centro de Neumologia Pediatrica; Luisa N. Borrell, City University of New York; Adam Davis, UCSF Benioff Children's Hospital, Oakland; Saunak Sen, University of Tennessee and Fred Lurmann, Sonoma Technologies, Inc.

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NHLBI TOPMed: Genetic Epidemiology Network of Arteriopathy (GENOA)

Support for GENOA was provided by the National Heart, Lung and Blood Institute (HL054457, HL054464, HL054481, HL119443, and HL087660) of the National Institutes of Health. WGS for "NHLBI TOPMed: Genetic Epidemiology Network of Arteriopathy" (phs001345) was performed at the Mayo Clinic Genotyping Core, the DNA Sequencing and Gene Analysis Center at the University of Washington (3R01HL055673-18S1), and the Broad Institute (HHSN268201500014C) for their genotyping and sequencing services. We would like to thank the GENOA participants.

NHLBI TOPMed: Genetic Epidemiology Network of Salt Sensitivity (GenSalt)

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NHLBI TOPMed: Genetics of Lipid Lowering Drugs and Diet Network (GOLDN)

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NHLBI TOPMed: Heart and Vascular Health Study (HVH)

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NHLBI TOPMed: Hypertension Genetic Epidemiology Network (HyperGEN)

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NHLBI TOPMed: The Jackson Heart Study

The Jackson Heart Study (JHS) is supported and conducted in collaboration with Jackson State University (HHSN268201800013I), Tougaloo College (HHSN268201800014I), the Mississippi State Department of Health (HHSN268201800015I/HHSN26800001) and the University of Mississippi Medical Center (HHSN268201800010I, HHSN268201800011I and

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NHLBI TOPMed: Multi-Ethnic Study of Atherosclerosis

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NHLBI TOPMed: Whole Genome Sequencing of Venous Thromboembolism (WGS of VTE)

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NHLBI TOPMed: MGH Atrial Fibrillation Study

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NHLBI TOPMed: Partners HealthCare Biobank

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NHLBI TOPMed: Study of African Americans, Asthma, Genes and Environment (SAGE)

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The SAGE study collaborators include Harold J. Farber, Texas Children's Hospital; Emerita Brigino-Buenaventura, Kaiser Permanente; Michael A. LeNoir, Bay Area Pediatrics; Kelley Meade, UCSF Benioff Children's Hospital, Oakland; Luisa N. Borrell, City University of New York; Adam Davis, UCSF Benioff Children's Hospital, Oakland and Fred Lurmann, Sonoma Technologies, Inc.

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NHLBI TOPMed: San Antonio Family Heart Study (WGS)

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NHLBI TOPMed: The Samoan Obesity, Lifestyle and Genetic Adaptations Study (OLaGA) Group

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Our study name: 'The Samoan Obesity, Lifestyle and Genetic Adaptations Study (OLaGA) Group'.

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NHLBI TOPMed: The Vanderbilt AF Ablation Registry

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NHLBI TOPMed: The Vanderbilt Atrial Fibrillation Registry

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NHLBI TOPMed: Novel Risk Factors for the Development of Atrial Fibrillation in Women

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NHLBI TOPMed: Women's Health Initiative (WHI)

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NHLBI TOPMed: GeneSTAR (Genetic Study of Atherosclerosis Risk)

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NHLBI TOPMed: Genetics of Sarcoidosis in African Americans (Sarcoidosis)

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