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Machine learning to optimize automated RH genotyping using whole-exome sequencing data

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Abstract:

Rh phenotype matching reduces but does not eliminate alloimmunization in patients with sickle cell disease (SCD) due to RH genetic diversity that is not distinguishable by serological typing. RH genotype matching can potentially mitigate Rh alloimmunization, but comprehensive and accessible genotyping methods are needed. We developed RHtyper as an automated algorithm to predict RH genotypes using whole-genome sequencing (WGS) data with high accuracy. Here, we adapted RHtyper for whole-exome sequencing (WES) data which are more affordable but challenged by uneven sequencing coverage and exacerbated sequencing read misalignment, resulting in uncertain prediction for 1) RHD zygosity and hybrid alleles, 2) RHCE*C versus RHCE*c alleles, 3) RHD c.1136C>T zygosity, and 4) RHCE c.48G>C zygosity. We optimized RHtyper to accurately predict RHD and RHCE genotypes using WES data by leveraging machine learning models and improved the concordance of WES with WGS predictions from 90.8% to 97.2% for RHD and 96.3 to 98.2% for RHCE among 396 patients in the Sickle Cell Clinical Research and Intervention Program (SCCRIP). In a second validation cohort with 3030 cancer survivors (15.2% Black or African Americans) from the St. Jude Lifetime Cohort Study (SJLIFE), the optimized RHtyper reached concordance rates between WES and WGS predications to 96.3% for RHD, and 94.6% for RHCE. In conclusion, machine learning improved the accuracy of RH predication from WES data. RHtyper has the potential, once implemented, to provide a precision medicine-based approach to facilitate RH genotype-matched transfusion and improve transfusion safety for patients with SCD.

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Clinical trial registration information (if any):

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Data Sharing Statement

WGS and WES data are available at St Jude Cloud (https://platform.stjude.cloud/data/cohorts) for the 396 patients with SCD from the SCCRIP study (accession number: SJC-DS-1006), and the 3030 cancer survivors from SJLIFE study (accession number: SJC-DS-1002).[18] The *RH* genotypes of the patients are included in supplemental Table 3 and Table 4. The source code and tutorial of RHtyper can be accessed via GitHub (https://github.com/disonchang/RHtyper).

Running title: Genotyping *RH* genes by RHtyper using WES data

KEY POINT

Machine learning optimized RHtyper for automated and accurate Rh blood group genotyping from whole-exome sequencing data.

Abstract

Rh phenotype matching reduces but does not eliminate alloimmunization in patients with sickle cell disease (SCD) due to *RH* genetic diversity that is not distinguishable by serological typing. *RH* genotype matching can potentially mitigate Rh alloimmunization, but comprehensive and accessible genotyping methods are needed. We developed RHtyper as an automated algorithm to predict *RH* genotypes using whole-genome sequencing (WGS) data with high accuracy. Here, we adapted RHtyper for whole-exome sequencing (WES) data which are more affordable but challenged by uneven sequencing coverage and exacerbated sequencing read misalignment, resulting in uncertain prediction for 1) *RHD* zygosity and hybrid alleles, 2) *RHCE*C* versus *RHCE*c* alleles, 3) *RHD* c.1136C>T zygosity, and 4) *RHCE* c.48G>C zygosity. We optimized RHtyper to accurately predict *RHD* and *RHCE* genotypes using WES data by leveraging machine learning models and improved the concordance of WES with WGS predictions from 90.8% to 97.2% for *RHD* and 96.3 to 98.2% for *RHCE* among 396 patients in the Sickle Cell Clinical Research and Intervention Program (SCCRIP). In a second validation cohort with 3030 cancer survivors (15.2% Black or African Americans) from the St. Jude Lifetime Cohort Study (SJLIFE), the optimized RHtyper reached concordance rates between WES and WGS predications to 96.3% for *RHD*, and 94.6% for *RHCE*. In conclusion, machine learning improved the accuracy of *RH* predication from WES data. RHtyper has the potential, once implemented, to provide a precision medicine-based approach to facilitate *RH* genotype-matched transfusion and improve transfusion safety for patients with SCD.

Introduction

Blood transfusion is an essential treatment for chronic anemia disorders including sickle cell disease (SCD) and thalassemia. Exposure to donor red blood cell (RBC) antigens can lead to alloimmunization and increase the risk of hemolytic transfusion reactions with subsequent transfusions.[1] Prophylactic matching for Rh (C, E or C/c, E/e) and K antigens lowers the risk of alloimmunization for patients with SCD and thalassemia but alloantibody formation against Rh blood group remains a challenge due to the genetically diverse *RH* genes of Black patients and blood donors.[2,3] The Rh blood group consists of five major antigens, D, C, c, E and e, and is encoded by highly homologous *RHD* and *RHCE* genes.[4] *RHD* and *RHCE* genes of individuals of African descent exhibit high diversity with single nucleotide polymorphisms (SNPs), insertions/deletions (indels), and structural variants. Approximately 450 *RHD* and 190 *RHCE* alleles have been identified (https://www.isbtweb.org/, accessed on 8/06/2023), and more than 50 Rh variant antigens have been described serologically. We found in our practice that 48-49% of patients with SCD and 41% Black blood donors in the US have a *RHD* or *RHCE* variant (excluding altered alleles of *RHD*10.00* or *RHD*DAU0* and *RHCE*01.01* or *RHCE*ce48C*), [5,6] and 7% of D-positive patients with SCD have a partial D.[7] In Brazil, 15% of patients with SCD and 8% of African Brazilian blood donors have both variant *RHD* and *RHCE* alleles.[8] These variant *RH* alleles encode proteins associated with loss of epitopes or expression of neo-epitopes. Individuals with variant *RH* alleles are at risk of alloimmunization when exposed to conventional or variant Rh antigens differing from their own. Since serological antigen typing cannot distinguish the presence of most variant Rh antigens,[2] *RH* genotyping and consideration of *RH* genotype matching can potentially improve resource allocation of valuable Black blood donors and avoid Rh alloimmunization.

Next generation sequencing (NGS) data such as whole-genome sequencing (WGS) and whole-exome sequencing (WES) offer comprehensive evaluation of the genome and have been used for *RH* genotyping.[9-13] Genotyping *RH* using NGS data are challenging since *RHD* and *RHCE* are duplicated genes and share 97% sequence identity. Sequencing reads from the highly homologous regions may map ambiguously, making it difficult to determine the true genomic origin of those reads. Therefore, analysis of NGS data from the *RH* loci requires sophisticated bioinformatic tools that can differentiate between true genetic variants and sequencing artifacts. We previously developed RHtyper for automated and accurate detection of the complex *RH* genotypes of Black or African American individuals using WGS data.[6] RHtyper relies on a Bayesian likelihood-based framework to infer *RH* genotypes directly after short read sequence alignment. Both sequence consistency at each SNP/indel and phase consistency across adjacent SNPs/indels are considered to improve prediction accuracy. RHtyper also incorporates coverage profiling to determine *RHD* zygosity and hybrid alleles, and can further define potential breakpoints of the hybrid *RH* alleles by the Circular Binary Segmentation algorithm. In a validation cohort of 57 patients with SCD, RHtyper achieved 100% accuracy for *RHD*, and 98.2% accuracy for *RHCE* when compared to the *RH* genotypes verified by multiple molecular methods. Upon application to the Sickle Cell Clinical Research and Intervention Program (SCCRIP) study cohort, RHtyper achieved high concordance rates of 98.3% with C serological typing (n=360 patients), 99.54% with D serological typing (n=219 patients).

WES is a focused and cost-effective strategy to identify exonic variations but has limitations we sought to overcome with machine learning. Sequencing coverage of WES is uneven because of variable sequencing read enrichment by capture oligonucleotides at different locations, leading to inaccurate prediction for copy numbers of exons and SNPs. WES data lack most intronic sequence markers essential for aligning sequencing reads, resulting in misalignments among highly homologous exons. Here, we adapted RHtyper for WES data by leveraging machine learning to address the uneven coverage and sequence misalignments, and improved WES-based *RH* genotyping substantially.

Methods

Patients

Existing WES and WGS data from 396 patients with SCD enrolled in the SCCRIP study and 3030 cancer survivors enrolled in St. Jude Lifetime Cohort Study (SJLIFE) at St Jude Children's Research Hospital (SJCRH) were included in this study. Of the 396 SCD patients, 56 patients had *RH* genotypes tested by standard *RH* genotyping method, SNP-based and targeted molecular assays, and confirmed by Sanger sequencing and NGS as previously described[6] and in Supplemental Methods. They were used to further verify the WES-predicted genotyping results. The SCCRIP is a lifetime longitudinal cohort study of patients with SCD, in which clinical information is prospectively collected and biologic samples are banked, including blood for genomics and proteomics studies. (NCT02098863)[14] The SJLIFE is a retrospective cohort study with prospective follow-up and ongoing accrual of oncology patients treated at SJCRH who are ≥18 years of age and ≥10 years post-diagnosis from their malignancy.[15] This study was approved by SJCRH institutional review board, and all participants or guardians provided written informed consent.

WES, WGS and serological typing

Genomic DNA was extracted from peripheral blood mononuclear cells by standard methods, and WGS and WES were performed at HudsonAlpha Institute for Biotechnology and the SJCRH Hartwell Center for Bioinformatics and Biotechnology as previously described.[14,16] Paired-end reads were aligned against the human genome (hg38) with the Burrows-Wheeler Aligner software package.[17] For patients in the SJLIFE cohort, serological typing of RhD only was performed.

Adjustment of RHtyper for WES data

An *RH* allele database was curated from the International Society of Blood Transfusion (ISBT) database and the now-retired NCBI Blood Group Antigen Gene Mutation (BGMUT) database as previously described.[6] The consolidated database included 419 *RHD* and 130 *RHCE* alleles annotated for genotype determination. Variants are determined according to conventional *RH* mRNA sequences (*RHD*, L08429, and *RHCE*, DQ322275), which differ from the reference genomic sequence (hg38) by 2 SNPs in the coding region (conventional *RHD* sequence, c.1136T, reference genomic sequence, c.1136C; conventional *RHCE* sequence, c.48G, reference genomic sequence, c.48C).

The WES-based RHtyper algorithm was developed according to WGS-based *RH* genotyping approach[6] with modification for WES data and by adding machine learning models to improve prediction accuracy (Figure 1). Specifically, the WES-based RHtyper algorithm consists of four main steps: 1) variant profiling for SNPs/indels and coverage alterations; 2) predicting *RHD* zygosity and hybrid alleles, *RHD* c.1136C>T and *RHCE* c.48G>C, and presence of *RHCE*C* or *RHCE*c* allele using established machine learning models; 3) refining the hybrid allele and hybrid breakpoint predictions using segmentation; 4) generating likelihood scores using genotypes and phased haplotype likelihoods to rank candidate allele pairs. Finally, the candidate allele pair with the highest likelihood scores is reported as the predicted genotype.

RH variant calling and coverage profiling

Variants were called via the SAMtools pileup method[17] using WES reads that met predefined read criteria (base read quality, ≥15; mapping read quality, ≥10; and average read quality, ≥15). Counts of A, T, G, and C nucleotides and indels were generated for each exonic position of *RHD*/*RHCE* genes. The exonic positions with variant allele-frequency > 10% were classified as heterozygous sites. SNPs and indels were annotated subsequently with encoded amino acid changes. *RHD/RHCE* coverage profiling was performed as previously described using WES data.[6]

Construction of machining learning models

The WGS-predicted genotypes served as control references.[6] Informative features were selected by the Boruta algorithm (10.18637/jss.v036.i11) based on per-base coverage and variant allele frequency. The selected features were then incorporated to construct XGboost models for model learning using 75% of the WES data from the SCCRIP study. The modified RHtyper was next validated using the remaining 25% of data from the SCCRIP study as well as a second patient cohort, the SJLIFE.

This study was approved by SJCRH institutional review board, and all participants or guardians provided written informed consent.

Results

Uneven sequencing coverage of WES data

The average *RH* sequencing coverage for 396 patients with SCD of the SCCRIP cohort was 56.3× for WES compared to 35.7× for WGS. WES coverage demonstrated high regional unevenness, the normalized coverage per *RHD* exon ranged from -6.09 ± 5.09 to 0.21± 1.60 (mean ± standard deviation, "0" representing 2 copies), and the normalized coverage per *RHCE* exon ranged from -0.80 ± 0.78 to 1.01± 0.35 (Figure 2 and Supplemental Table 1). In contrast, the normalized WGS coverage fluctuated less, ranging from -3.58 ± 3.50 to -0.40 ± 0.85 per *RHD* exon, and from -0.70 ± 0.33 to 0.35 ± 0.41 per *RHCE* exon. Notably, *RHD* coverage varied more than *RHCE* regardless of sequencing methods because *RHD* and *RHCE* have an identical exon 8, and most sequencing reads from exon 8 align to *RHCE*, reducing *RHD* exon 8 coverage markedly. The unevenness of WES coverage of *RH* genes affected prediction of zygosity of alleles and SNPs.

Limitation of RHtyper using WES data

Since RHtyper was initially designed for WGS data analysis, we first modified the algorithm for WES data to not rely on intronic markers for identification. *RHCE*C* can be predicted by WGS data with high confidence using a 109-bp insertion in *RHCE*C* intron 2. Since this intronic region is not covered by WES, *RHCE*C* was instead identified by increased coverage of *RHD* exon 2 since *RHCE*C* and *RHD* exon 2 are identical, and the reads from

*RHCE*C* typically align to *RHD*.[9] Of note, WES data cannot be used to identify alleles with only intronic variations.

We next determined *RH* genotypes using WES data for 396 SCD patients from the SCCRIP cohort, and all of whom are Black or African American. The concordance rates between WES and the previously reported WGS predictions[6] were 90.3% (715/792 alleles) for *RHD* and 96.3% (763/792 alleles) for *RHCE* (Figure 3A). The problematic determinations included 1) *RHD* zygosity and hybrid alleles, 2) *RHCE*C* versus *RHCE*c* alleles, 3) *RHD* c.1136C>T zygosity, 4) *RHCE* c.48G>C zygosity. *RHD* zygosity and hybrid alleles, and *RHCE*C* were predicted based on sequencing coverage of the whole gene (i.e., *RHD* zygosity) or certain exons of the gene (i.e., *RHCE* exon 4-7 for *RHD*03N.01* or *RHD*DIIIa-CEVS(4-7)-D, RHD* exon 2 for *RHCE*C*), which was less accurate with WES data due to the fluctuated sequencing coverage. *RHD* c.1136C>T (p. Thr379Met), located in exon 8, is the most common missense *RHD* SNP in patients with SCD and the characteristic SNP that defines the *RHD DAU* cluster.[6,19] Because the reference genomic sequence of *RHD* represents *RHD*10.00 or RHD*DAU0* with c.1136T, and the conventional *RHD* shares exon 8 with *RHCE* with c.1136C, almost all the sequence reads from conventional *RHD* align to *RHCE*, resulting in reduced coverage of *RHD* exon 8. To circumvent the skewed coverage of exon 8, *RHD* c.1136 C>T zygosity was determined for WGS data by dividing the reads containing the SNP by genome-wide average read coverage rather than position-specific read coverage. However, this approach was no longer reliable with WES data given the highly variable exome-wide sequencing coverage. *RHCE* c.48G>C (p. Trp16Cys) resides in exon 1 and is the most common missense *RHCE* SNP found in patients with SCD.[6] Also *RHCE*01.01* or *RHCE*ce48C* is as common as conventional *RHCE*01* or *RHCE*ce* allele in Black individuls.[5,6] The sequences of conventional *RHD* and *RHCE* exon 1 are highly homologous, differing only by one nucleotide, c.48C for *RHD* and c.48G for *RHCE*. Without the paired mate sequencing reads that cover the surrounding introns to differentiate *RHD* from *RHCE*, sequence reads with *RHCE* c.48G>C frequently misalign to *RHD*, resulting in erroneous G/C fraction and subsequent incorrect allele prediction.

Modification and validation of RHtyper for WES data

Given the low concordance between WES and WGS predictions, we sought to improve RHtyper by incorporating machine learning specific for the problematic alleles and SNPs. The SCCRIP cohort was used for training and validating the machine learning models because 1) all patients in the SCCRIP cohort are of African descent with highly diverse *RHD* and *RHCE* genes;[6] 2) despite Rh-phenotype matched blood transfusion, patients with SCD are still at high risk for Rh alloimmunization due to the genetic diversity of *RH* genes and will likely benefit the most from receiving *RH*-genotype matched blood transfusion.[2,5,20] A total of 1,547 informative features for *RHD* zygosity and hybrid alleles, 255 for *RHCE*C* versus *RHCE*c* allele differentiation, 240 for *RHD* c.1136C>T, and 253 for *RHCE* c.48G>C zygosity were selected to build machine learning models (Supplemental Figure 1). The *RH* genotypes predicted using WGS data were used as the reference genotypes because of the high accuracy.[6] We randomly selected 75% of WES data from the SCCRIP cohort for model training, and the remaining 25% for validation. Machine learning improved the concordance rates between WES and WGS predictions to 98.0% for *RHD* zygosity and hybrid alleles, 97.0% for the *RHCE*C* allele, 97.0% for *RHCE* c.48G>C zygosity, and 96.0% for *RHD* c.1136C>T zygosity. The overall concordance rates for the SCCRIP cohort were 97.2% (770/792 alleles) for *RHD*, 98.2% (778/792 alleles) for

RHCE (Figure 3B and Supplemental Table 2 and 3). The remaining discrepancies were due to, with substantially fewer numbers though, *RHD* zygosity and hybrid alleles (6 alleles, 0.8% of total 792 *RHD* alleles), *RHCE*C* versus *RHCE*c* (8 alleles, 1% of total 792 *RHCE* alleles; 6 *RHCE*C* mis-predicted as *RHCE*c*, 0.8% of total *RHCE* alleles; 2 *RHCE*c* as *RHCE*C*, 0.2% of total *RHCE* alleles), *RHCE* c.48G>C zygosity (5 alleles, 0.6%), *RHD* c.1136C>T zygosity (9 alleles, 1.1%), and other SNP discrepancies (*RHD*, 7 alleles, 0.9%; *RHCE*, 1 allele, 0.1%).

In the SCCRIP cohort, the *RH* genotypes of 56 patients were also determined by standard *RH* genotyping methods including *RH* SNP-array, targeted molecular assays, and verified by Sanger sequencing or a 2nd independent NGS as described previously[6] and in Supplemental Methods. Compared to the verified genotypes, the modified RHtyper using WES data achieved 98.2% (110/112 alleles) accuracy for *RHD* and 94.6% (106/112 alleles) accuracy for *RHCE* alleles (Table 1). Of note, none of the erroneous predictions would have led to increased risk of Rh alloimmunization. One erroneous prediction where patient 1 with “RhC” (*RHCE*02/RHCE*01.20.01 or RHCE*Ce/RHCE*ce733G*) was misidentified by WES as “Rhc” (*RHCE*01/ RHCE*01.20.02* or *RHCE*ce/RHCE*48C733G*) could have resulted in the C-positive patient receiving C-negative blood unnecessarily.

Further validation of the modified RHtyper in the SJLIFE cohort

Next, we further validated the modified RHtyper in a second available patient cohort, SJLIFE consisting of 3030 cancer survivors. Among 2716 patients with racial information, 84.6% (2298) are White, 15.2% (413) Black or African American, 0.11% (3) Asian, 0.04% (1) American Indian or Alaska Native, and 0.04% (1) Native Hawaiian or Other Pacific Islander.

The concordance rates between WES and WGS predictions were 96.3% (5837/6060 alleles) for *RHD*, and 94.6% (5734/6060 alleles) for *RHCE* (Figure 4 and Supplemental Table 4). Discrepancies included *RHD* zygosity and hybrid alleles (159 alleles, 2.6% of total 6060 *RHD* alleles), *RHCE*C* versus *RHCE*c* differentiation (263 alleles, 4.3% of total 6060 *RHCE* alleles; 237 *RHCE*C* mis-predicted as *RHCE*c*, 3.9% of total *RHCE* alleles; 26 *RHCE*c* as *RHCE*C*, 0.4% of total *RHCE* alleles), *RHCE* c.48G>C zygosity (37 alleles, 0.6%), *RHD* c.1136C>T zygosity (17 alleles, 0.3%), and SNPs and other discrepancies (*RHD*, 47 alleles, 0.8%; and *RHCE*, 26 alleles, 0.4%). For 1036 patients with blood type information, the predicted RhD serological types using WES data were 99.8% (1034/1036 patients) consistent with the clinical serology results; of note, this comparison only assessed whether RHtyper could correctly predict the presence or absence of RhD. The predicted frequency of C antigen was 65.23% (1499/2298 patients) per WGS and 58.96% (1355/2298 patients) per WES for White patients, and 23.24% (96/413 patients) per WGS and 24.21% (100/413 patients) per WES for Black or African American patients, consistent with the known racial distribution (68% of White people and 27% of Black people).[21]

The modified WES-based RHtyper was trained primarily using data from Black or African American patients, while the majority of patients in the SJLIFE cohort were White for whom the frequency of *RH* variation is ~1-2%.[21] Therefore, we compared the concordance rates of White versus Black or African American patients in the SJLIFE cohort (Figure 5). Discrepancies were significantly higher among White patients for *RHD* zygosity and hybrid alleles (127 alleles or 2.8% of *RHD* alleles in White patients versus 11 alleles or 1.3% of *RHD* alleles in Black or African American patients, p = 0.0157), and *RHCE*C* versus *RHCE*c* differentiation (227 alleles or 5.0% of *RHCE* alleles in White patients versus 8 alleles or 1.0% of *RHCE* alleles in Black or African American patients; p<0.0001). In contrast, discrepancy for *RHD* c.1136C>T zygosity was significantly higher in Black or African American patients (10 alleles or 1.2% of *RHD* alleles in Black or African American patients versus 1 allele or 0.02% of *RHD* alleles in White patients; p<0.0001), although the overall numbers of discrepant alleles were very low.

Discussion

The WGS-based RHtyper relies on sequencing coverage profiles to predict the zygosity of alleles and SNPs.[6] This approach alone was less accurate for analyzing WES data due to the uneven sequencing coverage and misalignment of sequencing reads. To improve the prediction accuracy with WES data, we optimized RHtyper by leveraging machine learning to target the four most affected SNPs and alleles including 1) *RHD* zygosity and hybrid alleles, 2) *RHCE*C* versus *RHCE*c* alleles, 3) *RHD* c.1136C>T zygosity, and 4) *RHCE* c.48G>C zygosity. Machine learning substantially increased the concordance of WES- with WGS-predicted *RH* genotypes when applied to two independent large patient cohorts, SCCRIP and SJLIFE, but a few limitations remained.

Manual or automated genotyping of *RHD* and *RHCE* from targeted exome sequencing and WES data has been performed by multiple groups.[9,10,22-25] Prediction of *RHD* zygosity and hybrids, and *RHCE*C* versus *RHCE*c* alleles by sequencing coverage has consistently been difficult with WES data. Schoeman et al reported that the sensitivity to detect a deletion in *RHD* and *RHCE* was 89.8%, and only 52.8% for duplications using sequencing coverage alone (n=28).[25] To overcome this limitation, Chou et al. and Lane et al. determined *RHD* zygosity using *RHCE* as a control since nearly all individuals have two copies of *RHCE,* and *RHCE*C* identification was based on decreased read coverage of *RHCE* exon 2 compared to *RHCE* exons 1 and 3.[9,13] Chou et al. reported that the approaches improved the concordance rate to 98% (n=54).[9] Lane et al. developed the first automated algorithm for RBC antigen genotyping using WES data.[13] By using copy number correction factors calculated from 20 individuals of known *RHD* zygosity and C/c antigen status to normalize the sequencing coverage of each exon, the authors were able to correctly genotype the remaining 55 individuals. The improvement strategies utilized by those studies involved creating pre-determined rules based on data from a small cohort of individuals. However, this approach may not be comprehensive enough to capture all the necessary information for accurate prediction in a large number of individuals. WES data were also not reliable in predicting *RHCE* c.48G>C and *RHD* c.1136C>T due to misalignment of sequencing reads.[9,25] The algorithm created by Lane et al was able to detect *RHD* c.1136C>T but could not distinguish homozygous from heterozygous ones.[13]

We optimized RHtyper for WES data by using machine learning. The learning process allows for incorporation of diverse informative features and has been applied to complicated and high-dimensional data including genomic sequencing data. It enables accurate predictions based on automated data learning rather than simple rule-based classification. In our study, informative features of per-base coverage and variant allele frequency from hundreds to thousands of exonic positions were used to identify the problematic SNPs and alleles. Training with almost 300 SCD patients from the SCCRIP cohort allowed recognition of intricate patterns for accurate prediction. Machine learning markedly improved the concordance rates between

WES and WGS predictions to 97.2% for *RHD* and 98.2% for *RHCE* in the SCCRIP cohort (n = 396), and 96.3% for *RHD* and 94.6% for *RHCE* in the SJLIFE cohort (n = 3030). By using similar machine learning approaches, RHtyper can be extended to analyze other blood group proteins encoded by highly homologous genes, for example, the MNS blood group.

Discordant predictions between WES and WGS remained despite machine learning. *RHD* zygosity and hybrid alleles and *RHCE*C* versus *RHCE*c* alleles contributed to most of the discrepancies. Discordant *RHD* zygosity and hybrid allele calling occurred more often to patients with hemizygous *RHD* deletion or heterozygous *RHD* hybrid alleles, which require sufficient and even coverage for accurate identification and could remain challenging for certain patients even with machine learning. Since the sequencing coverage of *RHD* exon 2 is critical in differentiating *RHCE*C* versus *RHCE*c* alleles, we initially suspected that the coverage might be erroneous due to misalignment mediated by SNPs unique in certain patients. However, comparison of *RHD* exon 2 and its surrounding intron sequences (50 bps into the surrounding introns) between patients with and without *RHCE*C* versus *RHCE*c* discrepancy revealed no SNPs that would have led to misalignment (data not shown). Furthermore, White patients in the SJLIFE cohort were more likely to have discordant predictions of *RHD* zygosity and hybrid alleles and *RHCE*C* versus *RHCE*c* alleles than Black or African American patients. The skewed discordance could be due to higher frequencies of D negative and C positive status in White (15% and 68% respectively) than in Black or African American patients (8% and 27% respectively).[21] Racial difference between the training and validation cohorts could also provide an explanation as all patients in the SCCRIP training cohort are Black or African American and 84.6% of the patients in the SJLIFE validation cohort are White. It is possible that individuals from different races may have slightly different sequencing coverage patterns, or the informative features used to identify and/or differentiate those alleles vary with race, for which future studies are warranted. Additional training using WES data from White individuals and individuals of other racial and ethical groups is needed to further improve RHtyper accuracy.

Clinical implementation of RHtyper may become increasingly relevant as more patients with chronic diseases are being interrogated by WES or WGS. It provides an analysis tool for data that may already exist or are obtained for other clinical care. *RH* genotyping can enhance transfusion safety by facilitating anti-Rh antibody identification and/or in some cases, improve prophylactic RBC matching strategies. For example, for patients with the hybrid alleles of *RHD*01N.03* or *RHD*DIIIa-CEVS (4-7)-D*, *RHCE*02.10.01* or *RHCE*CeRN*, which encode partial C antigen, and no conventional *RHCE*Ce* or *RHCE*CE* allele, we recommend transfusion with C negative RBCs to prevent anti-C formation.[26] Genotyping blood donors, particularly frequent Black donors who support C/E/K-matched RBCs for patients with SCD, may facilitate *RH* genotype-matched blood transfusion and improve transfusion safety in the future. RHtyper achieved high concordance rates in two large validation cohorts after incorporating the machine learning models but was not 100% accurate. One limitation is that RHtyper may mis-predict *RHCE*C* and *RHCE*c* with WES data. Misidentification of *RHCE*C* as. *RHCE*c* may result in C positive patients receiving C negative blood, which would not cause any harm to the patient, but from a resource perspective, it would be poor allocation of C antigen negative units. Conversely, misidentification of *RHCE*C* as. *RHCE*c* in blood donors may result in exposing C negative recipients to C positive blood and potential anti-C formation. Therefore, the use of RHtyper for *RH* genotyping blood donors would need to be combined with other testing such as standard serologic typing. For clinical application, additional training and validation using samples from multiple racial groups with *RH* genotypes verified by *RH* SNP-array, Sanger sequencing, and other molecular methods as well as serological tests are essential to further optimize RHtyper predictions.

There are limitations to our study. First, we used WGS-predicted genotypes by RHtyper as the reference. This seemed justified as we previously demonstrated that the WGS-predicted genotypes were highly accurate compared to genotypes verified by multiple molecular methods, and serological types for D and C/c antigens.[6] Second, the SJLIFE cohort was only serologically typed for D antigen, and thus, concordance with C antigen typing was not possible. However, the prevalence of C antigen in White and Black or African American patients derived from WGS and WES data were consistent with known frequencies, indicating the genotyping results were likely accurate.[21]

In conclusion, we optimized RHtyper for WES data by adding machine learning to overcome the variable sequencing coverage and misalignment associated with WES data. The optimization improved *RH* genotyping accuracy and extended the application spectrum of RHtyper to include the more widely available WES data.

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Author contributions

Contributions: J.S.H., M.J.W., C.M.W., S.T.C., and Y.Z. designed the research. T.C.C. and G.W. developed and modified RHtyper. J.Y. performed confirmatory Sanger sequencing. J.S.H, and M.J.W. and Z.W. provided patient samples and sequencing data. T.C.C., C.M.W., S.T.C., and Y.Z. wrote the manuscript.

Conflict of interest: The authors have no competing financial interests.

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Table 1. Discrepancies between WES-predicted genotypes and genotypes determined by *RH* SNV-array and PCR-based assays in a validation cohort with 56 SCD patients.

Patient	*RH* Allele	*RH* SNV-array and PCR-based assays	WES-predicted genotypes	Confirmation methods
1	D	*RHD*01*	*Same as left*	–
	D	*Deletion*	*Same as left*	–
	CE	*RHCE*02 (RHCE*Ce)*	*RHCE*01 (RHCE*ce)*	Serology
	CE	*RHCE*01.20.01 (RHCE*ce733G)*	*RHCE*01.20.02 (RHCE*ce 48C, 733G)*	Serology
2	D	*RHD*	*RHD*10.00 (RHD*DAU0)*	Sanger sequencing
	D	*RHD*	*RHD*10.00 or (RHD*DAU0)*	Sanger sequencing
	CE	*RHCE*02 or RHCE*Ce*	*Same as left*	–
	CE	*RHCE*01.20.01 (RHCE*ce733G)*	*RHCE*01.20.02 (RHCE*ce 48C, 733G)*	Sanger sequencing
3	D	*RHD*01*	*Same as left*	–
	D	*Deletion*	*Same as left*	–
	CE	*RHCE*01 (RHCE*ce)*	*An extra RHCE c.105C>T identified*	Sanger sequencing
	CE	*RHCE*01.20.02 (RHCE*ce 48C, 733G)*		Sanger sequencing
4	D	*RHD*01*	*Same as left*	–
	D	*Deletion*	*Same as left*	–
	CE	*RHCE*01 (RHCE*ce)*	*Same as left*	–
	CE	*RHCE*01 (RHCE*ce)*	*RHCE*01.01 (RHCE*ce48C)*	Sanger sequencing

Figure legends

Figure 1. Modification of RHtyper for WES data by adding machine learning. The WES-based RHtyper algorithm consists of four main steps: 1) variant profiling for SNPs/indels and coverage alterations; 2) predicting *RHD* zygosity and hybrid alleles, *RHCE*C* and *RHCE*c*, the zygosity of *RHD* c.1136C>T and *RHCE* c.48G>C by machine learning models; 3) refining the hybrid allele and hybrid breakpoint predictions using segmentation; 4) generating likelihood scores using genotypes and phased haplotype likelihoods to rank candidate allele pairs. Finally, the candidate allele pair with the highest likelihood scores is considered as the predicted genotype. WES, whole exon sequencing; BAM, binary alignment map; QC, quality control.

Figure 2. Uneven sequencing coverage of *RH* genes by WES compared to WGS. Sequencing coverage is normalized by log2 transformation of the ratio between each exon and the sample's average coverage. A value of "0" represents 2 copies. Exons are differentiated by colors. Lines represent mean sequencing coverage. Shadows represent standard deviation.

Figure 3. For the 396 SCD patients enrolled in the SCCRIP study, machine learning increased the overall concordance rates between WES- and WGS-predicted *RH* genotypes from 90.3% for *RHD* and 96.3% for *RHCE* (A) to 97.2% for *RHD*, 98.2% for *RHCE* (B). The number and percentage of concordant alleles and various types of discordant alleles are shown in parentheses. SNP, single nucleotide polymorphism.

Figure 4. The modified RHtyper achieved high concordance rates between WES- and WGS-predicted *RH* genotypes of 96.3% for *RHD* and 94.6% for *RHCE* in the SJLIFE cohort consisting

of 3030 cancer survivors. The number and percentage of concordant alleles and various types of discordant alleles are shown in parentheses. SNP, single nucleotide polymorphism.

Figure 5. Discordance rates of the trained SNPs/alleles among White and Black or African American patients in the SJLIFE study. NS represents non-significant; * represents p<0.05; **** represents p<0.001.

Figure 1

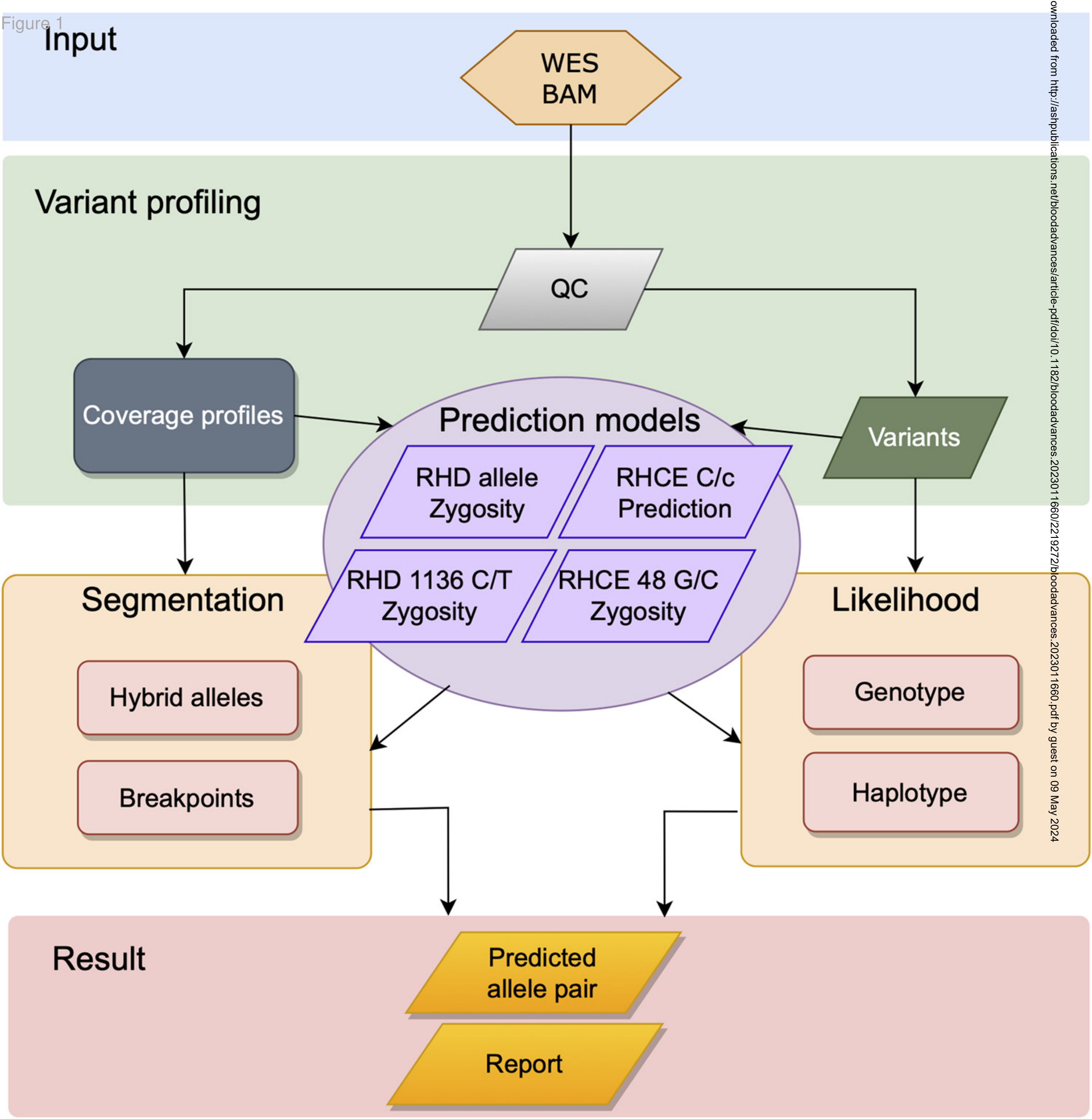

Input
WES BAM
Variant profiling
QC
Coverage profiles
Variants
Prediction models
RHD allele Zygosity
RHCE C/c Prediction
RHD 1136 C/T Zygosity
RHCE 48 G/C Zygosity
Segmentation
Hybrid alleles
Breakpoints
Likelihood
Genotype
Haplotype
Result
Predicted allele pair
Report

Figure 2

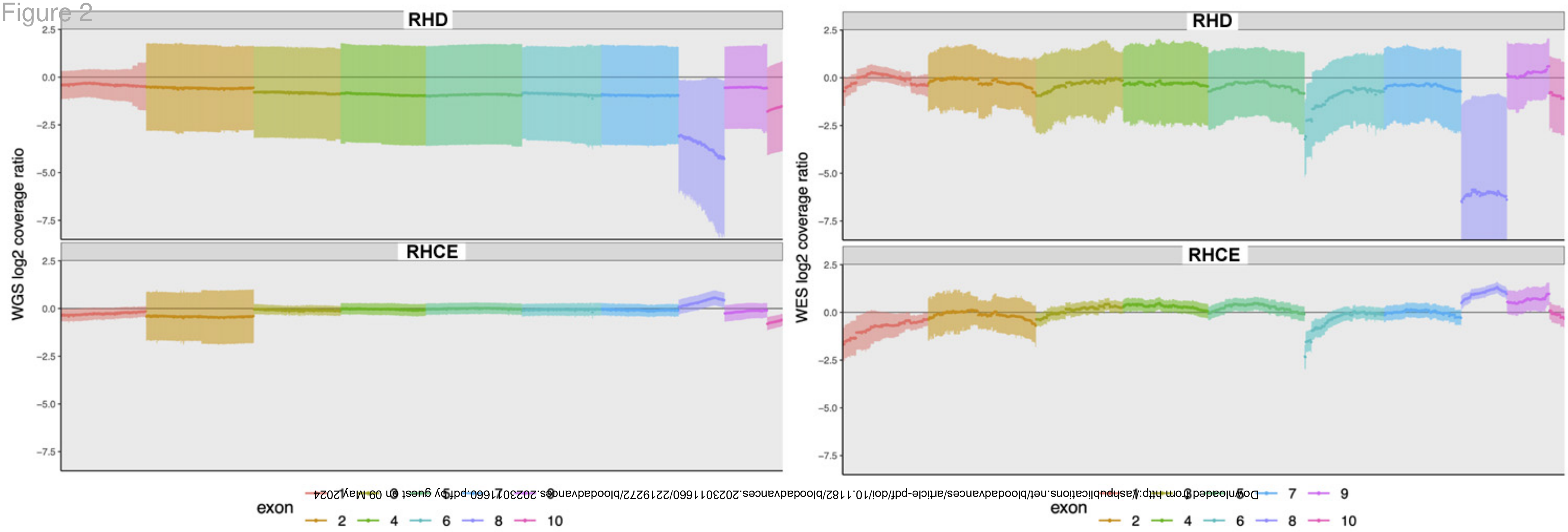

RHD
RHCE
WGS log2 coverage ratio
2.5
0.0
-2.5
-5.0
-7.5
exon
1
2
3
4
5
6
7
8
9
10
RHD
RHCE
WES log2 coverage ratio
2.5
0.0
-2.5
-5.0
-7.5
exon
1
2
3
4
5
6
7
8
9
10

Figure 3

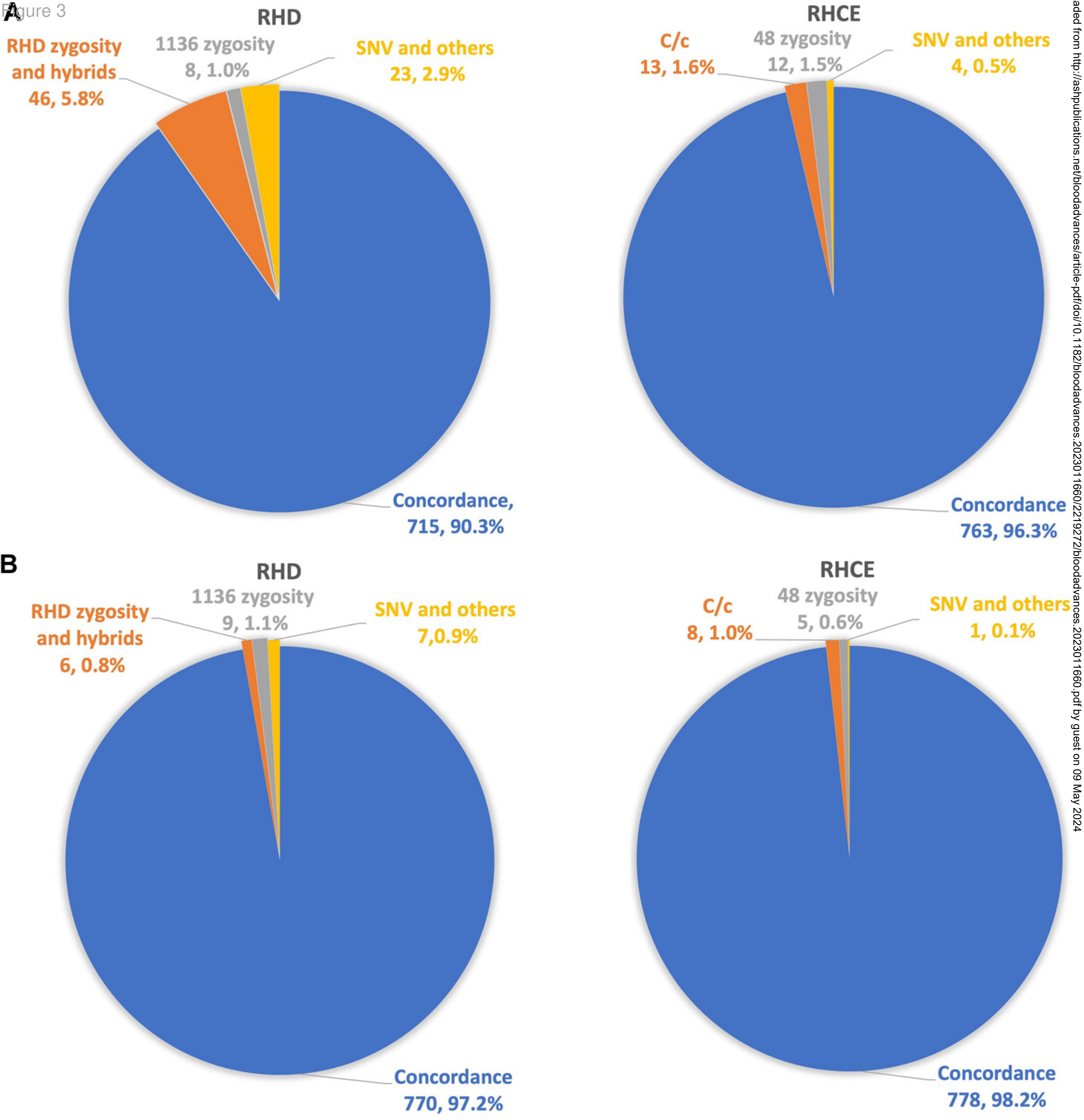

A
RHD
RHD zygosity and hybrids 46, 5.8%
1136 zygosity 8, 1.0%
SNV and others 23, 2.9%
Concordance, 715, 90.3%
RHCE
C/c 13, 1.6%
48 zygosity 12, 1.5%
SNV and others 4, 0.5%
Concordance 763, 96.3%
B
RHD
RHD zygosity and hybrids 6, 0.8%
1136 zygosity 9, 1.1%
SNV and others 7,0.9%
Concordance 770, 97.2%
RHCE
C/c 8, 1.0%
48 zygosity 5, 0.6%
SNV and others 1, 0.1%
Concordance 778, 98.2%

Figure 4

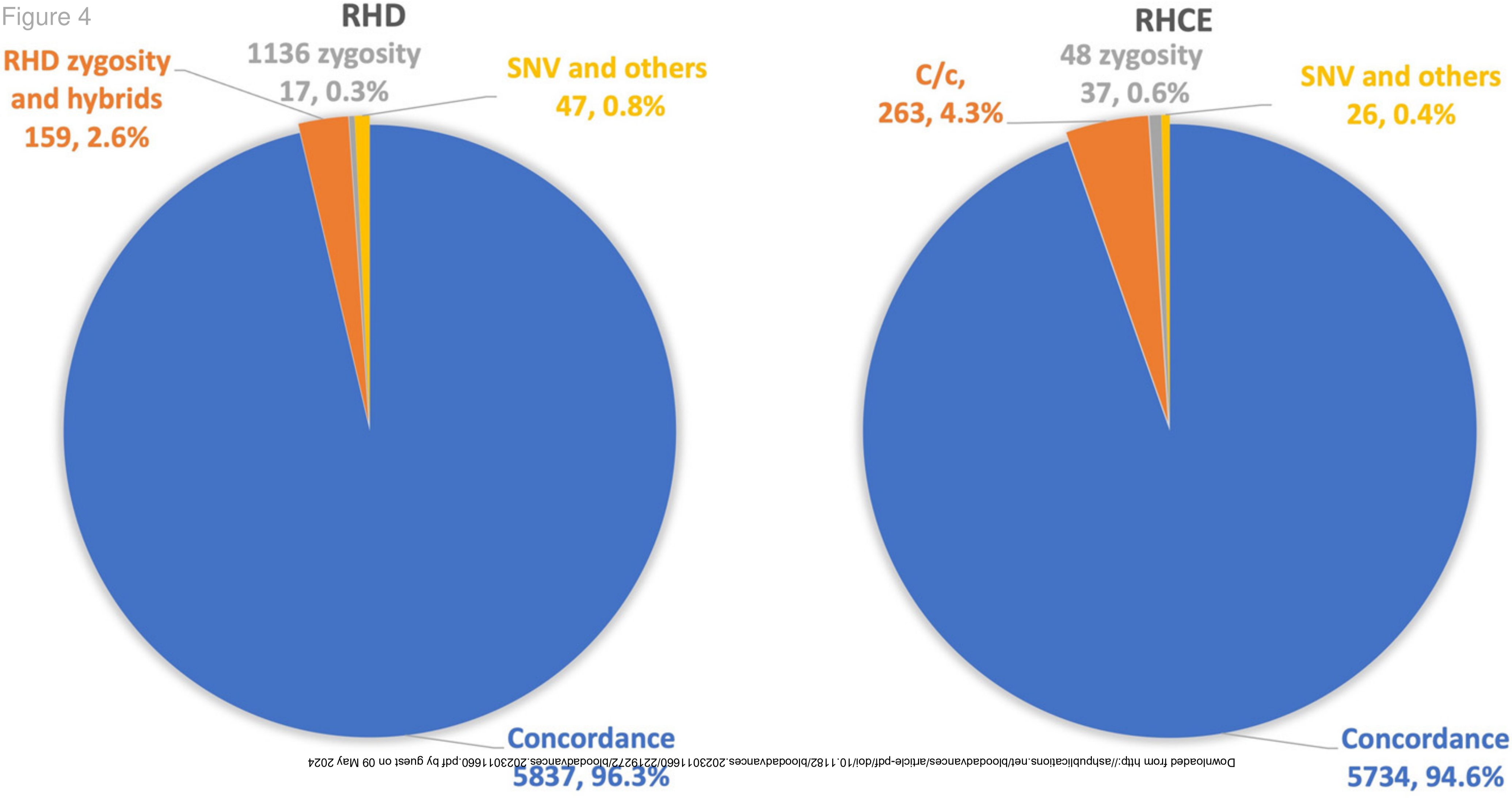
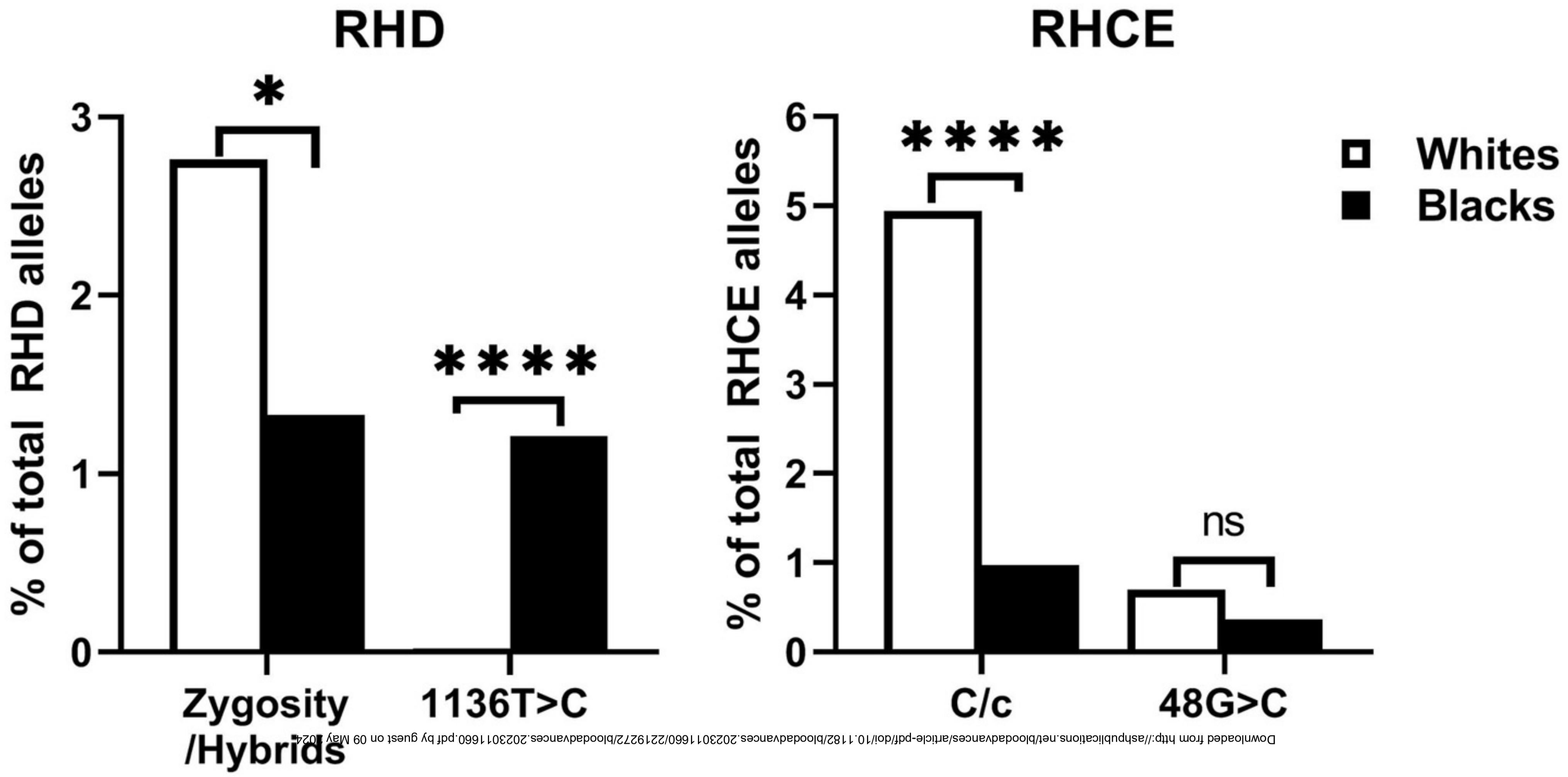

RHD
RHD zygosity and hybrids 159, 2.6%
1136 zygosity 17, 0.3%
SNV and others 47, 0.8%
Concordance 5837, 96.3%
RHCE
C/c, 263, 4.3%
48 zygosity 37, 0.6%
SNV and others 26, 0.4%
Concordance 5734, 94.6%

Figure 5


RHD
% of total RHD alleles
0
1
2
3
*
****
Zygosity /Hybrids
1136T>C
RHCE
% of total RHCE alleles
0
1
2
3
4
5
6
****
ns
C/c
48G>C
Whites
Blacks