RFPRED: A RANDOM FOREST APPROACH FOR PREDICTION OF MISSENSE VARIANTS IN HUMAN EXOME

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Exome sequencing is becoming a standard tool for gene mapping of genetic diseases. Given the vast amount of data generated by Next Generation Sequencing techniques, identification of disease causal variants is like finding a needle in a haystack. The impact assessment and the prioritization of potential pathogenic variants are expected to reduce work in biological validation, which is long and costly.

One of the possible approaches to determine the most probable deleterious variants in individual exomes is to use protein function alteration prediction. We propose in this paper to use a machine learning approach, the random forest to build a new meta-score based on five previously described scores (SIFT, Polyphen2, LRT, PhyloP and MutationTaster) and compiled in the dbNSFP database.

The functional meta-score was trained on a dataset of 61 500 non-synonymous Single Nucleotide Polymorphisms (SNPs). The random forest method (rfPred) appears to be globally better than each of the classifiers separately or in combination in a logistic regression model, and better than a newly described score (CADD) on independent validation sets.

RfPred scores have been pre-calculated for all the possible non-synonymous SNPs of human exome and are freely accessible at the web-server <http://www.sbim.fr/rfPred/>

Keywords: genetic variant prediction; random forest; deleteriousness score; sequence analysis; SNP

1. Introduction

Exome sequencing is a recent and important innovation for the exploration of patients affected by a genetic disease, in particular in situations when the other approaches have failed. The difficulty is often to find the disease causal variant(s), and it may be useful to focus on computational approaches like functional alteration predictors, to synthesize all available a priori information for variants prioritization before further functional studies.

Many different methods have been developed and published over the past fifteen years, each of these has distinct advantages and disadvantages, but none can be considered as the gold standard¹²³. The prediction scores of some of these methods have been compiled in the dbNSFP database for all known protein coding genome positions⁴. Besides, Li and colleagues proposed to combine five of them in a logistic regression framework⁵ in order to globally improve predictive performance in comparison with individual scores.

The idea of this contribution is twofold. First, we propose to combine the scores of different functional predictors using a machine learning method (random forest) that should be more suited to the nature of the problem than the logistic regression framework of Li and colleagues⁵. The performance of this method will be compared to the five models taken separately, to a logistic regression framework and to the recently published CADD method⁶. Second, we make available to the scientific community the pre-calculated prediction scores of our approach for all possible missense SNPs in the human exome.

2. Materials and Methods

2.1 Data collection for model building

First, we constructed a Single Nucleotide Variant (SNV) dataset with a status variable, taking values “neutral” or “deleterious”, in order to build the prediction model. For the deleterious variants, we used the OMIM database (Online Mendelian Inheritance in Man)⁷ - 23/09/2001 version- available at <https://main.g2.bx.psu.edu/library>, which inventories variants and phenotypes associated with Mendelian diseases. It contains 9130 human genome variants using the hg19 map. These variants will be considered as deleterious.

To build an assumed neutral variant set, we started with the 1000 genome database available via ANNOVAR⁸ <http://www.openbioinformatics.org/annovar/> (version from November 2010 updated in June 2011), which inventories the genetic data of supposed healthy subjects. Among these data, we selected the missense variants with the already existing hg19_avsift filter of ANNOVAR, and those with an allele frequency < 1% in the population (rare variants); their neutral nature is not obvious and corresponds to the reality with which researchers are faced.

For each of these variants, a score has been attributed with the five following methods: SIFT (released August 2011)⁹, Polyphen2 (HumDiv classifier model v2.1.0)¹⁰, Mutation Taster (released March 2010)¹¹, LRT (released November 2009)¹² and PhyloP¹³ thanks to the dbNSFP public database <https://sites.google.com/site/jpopgen/dbNSFP>.⁴ This database contains all possible SNPs within human genome coding regions, which have been determined by the CCDS project¹⁴, and for each of the 87 million SNPs, the scores of the five predictors have been pre-calculated and made available. The scores are used raw (directly calculated by the softwares) or processed such that the pathogenicity probability increases with increasing score.

We have kept in this training dataset only variants with the five available scores, which can be reduced to 6 254 deleterious SNPs and 55 223 neutral SNPs.

2.2 Data Collection for external validation

In order to evaluate the prediction model on independent datasets, we have used 2 general and 2 more precise datasets:

- A published variant dataset – EXOVAR – already used to evaluate similar methods (available at <http://statgenpro.psychiatry.hku.hk/limx/kggseq/download/ExoVar.xls>) including both 4752 neutral (from 1000 Genomes Project¹⁵, with a derived allele frequency <1%) and 5340 disease causing variations (with known effects on the molecular function causing human Mendelian diseases from the UniProt database)¹⁶, but only 1740 neutral and 3601 disease causing variants not included in our learning dataset with all necessary scores available.
- A validation dataset composed of 1100 pathogenic non-synonymous variations coming from ClinVar database (annotated as “Pathogenic”) and of 5412 variations coming from 1000 Genomes Project¹⁵

with a minor allele frequency between 5% and 20% , considered as non-deleterious and for which the 5 predictors give a score. None of these variations are part of our learning dataset.

Two more specific missense genetic variant datasets coming from two genes having many deleterious or polymorphic known variants:

- The first one is the *COL4A5* gene on the X chromosome which is composed of 51 exons making up a total length of 257kb. Many variants in this gene are implied in the Alport syndrome [MIM 301050], but SNPs without known disease association are also reported in public databases. After having discarded the SNPs included in our learning dataset, this first validation dataset contains:
 - 34 neutral variants coming from the dbSNP database – build 137 ¹⁷, with the “clinical significance” annotation in the database different from “probable pathogenic”
 - 168 deleterious variants coming from dbSNP database - build 137 also, annotated “probable pathogenic”, and from HGMD database (public version) ¹⁸.
- The second analyzed gene is the *COL7A1* gene on the chromosome 3 which is composed of 118 exons making up a total length of 32kb. Some variants in this gene are associated in the dystrophic epidermolysis bullosa [MIM 131750]. Our validation dataset contains:
 - 325 neutral variants coming from the dbSNP database – build 137, with the “clinical significance” annotation in the database different from “probable pathogenic”
 - 162 deleterious variants coming from dbSNP database – build 137 annotated “probable pathogenic” and the col7info database ¹⁹.

2.3 Statistical method

Relationships between the five scores on the training dataset were analyzed by computing the Spearman's rank correlation matrix. The meta-score rfPred was derived from the five individual scores using the predictions of a random forest model²⁰.

Briefly a random forest is based on a set of classification trees trained on a random subset of observations and variables of the complete dataset. Each tree votes for a given class, here either neutral or deleterious variant, and the final classification is based on the trees vote's majority. In fact, more precise information is provided by the proportion of tree votes in favor of the deleterious class and it can be used as a credibility index of the classification. So we used this vote proportion as a predictive meta-score of functional alteration.

We generated a random forest model composed of 5 000 trees based on two random scores among the five available for splitting at each tree node, using the model deviance as classification criterion. Each tree was trained against an equal sampling of 2000 variants of each class (deleterious and neutral).

All the analyses were made with R software version 2.15.0, and in particular with the package named “randomForest” ^{21,22}.

2.4 Statistical model evaluation

To compare the predictors' performances with the rfPred one, we have used the individual prediction scores (Polyphen2, SIFT, LRT, PhyloP and MutationTaster) coming from dbNSFPv2.0 ²³ and CADD scores computed via its web-interface⁶. We have then computed the ROC curves and the areas under the curves of the different classifiers thanks to the R package “Hmisc”²⁴ on the learning dataset and on the validation datasets.

In order to have a more useful comparison between the classifiers, we have considered also two particular scenarii corresponding to the reality faced by molecular biologists:

In the one hand, the classification model is used on a very long variants list coming directly from the exome alignment on the reference genome in order to establish a list a potentially pathogen variants. The purpose in this case is to keep all potentially deleterious variants and not to exclude any potential candidate. We favor in this case the sensitivity to the detriment of the specificity.

In the other hand, a variant selection process has been made beforehand by other methods (typically existing methods in pipelines of next-generation sequencing platforms and/or prior knowledge about the genetic basis of the disease), and the classification model will be used in a second time to prioritize the work areas. The goal is to minimize false positives variant rates to concentrate biological efforts on variants with a high probability of pathogenicity. We thereby favor specificity.

For these specific scenarii, partial AUC (Areas Under Curve) restricted to False Negative Rates < 10% for the first scenario and to False Positive Rates < 10% for the second one, as well as the ratio $\frac{\partial AUC}{\max(\partial AUC)}$ (partial area index) have been calculated.

MutationTaster has not been evaluated on the ClinVar-1000Genomes dataset because a very large part of the variants have been used to train the method.

2.5 Missing data handling

For some exome positions, one or several pre-calculated scores were missing in dbNSFP 2.0²³. Because we wanted to be able to use rfPred even if one of the score is missing for a variant position, we have imputed the missing score value by a random forest approach implemented in the R package “yaImpute”²⁵, based on a k-NN algorithm. We have used k=1 in our procedure.

3. Results

Spearman’s correlation matrix for the individual predictors is given in Table 1. It shows low to moderate correlation between scores (0.18 for the minimal and 0.66 for the maximal correlation) on the learning dataset, indicating that the information contained in the five prediction scores should not be completely redundant and that a combination of them could therefore be pertinent.

Table 1: Spearman correlation coefficients matrices on learning dataset with OMIM deleterious variants and neutral variants with allele frequency < 1%

Datasets		PhyloP	SIFT	Polyphen2	LRT	MutationTaster
Neutral Variants From 1000 genomes (Allele frequency < 1%)	PhyloP	1	0.28	0.43	0.6	0.47
	SIFT		1	0.58	0.28	0.32
	Polyphen2			1	0.45	0.47
	LRT				1	0.66
	MutationTaster					1
Deleterious Variants From OMIM	PhyloP	1	0.18	0.22	0.35	0.23
	SIFT		1	0.52	0.33	0.38
	Polyphen2			1	0.43	0.42
	LRT				1	0.53
	MutationTaster					1

3.1 Model prediction performance

The random forest prediction model (rfPred) has an AUC of 0,849 on the “Out of bag” learning dataset (unbiased AUC), whereas the best individual predictor on the same dataset, MutationTaster, obtains only 0,804 of AUC. The simple logistic model meanwhile obtains a maximal AUC of 0,827, even if the AUC is computed on the totality of the sample used to build the model. About CADD, it reaches the AUC of 0,770. We can note that rfPred ROC curve lies above all the others on the whole curve. (Figure 1)

The variable importance is measured in the rfPred model by the mean decrease in Gini coefficient.

It is a measure of how each variable contributes to the homogeneity of the nodes and leaves in the resulting random forest. Each time a particular variable is used to split a node, the Gini coefficient for the child nodes are calculated and compared to that of the original node. The Gini coefficient is a measure of homogeneity from 0 (homogeneous) to 1 (heterogeneous). The changes in Gini are summed for each variable and normalized at the end of the calculation. Variables that result in nodes with higher purity have a higher decrease in Gini coefficient.

Table 2 indicates that the two main predictors are Mutation Taster and SIFT scores for rfPred model. This is consistent with the layout of the observed ROC curves.

Table 2: Variables importance in rfPred model

Variable	Mutation Taster	SIFT	PhyloP	LRT	Polyphen 2
Mean Gini coefficient Decrease	607	364	340	306	299

Fig 1: Classifiers' ROC curves on learning dataset

In order to address the two above named specific situations (avoiding missing a potential deleterious variant, or on the contrary, identifying only variants with a very high pathogenicity probability), we have calculated a partial AUC for very high sensitivity or specificity (between 0,9 and 1), and compared these results with the partial area index. These indices can be interpreted as the mean sensitivity (specificity) of each classifier for the studied specificities (sensitivities)²⁶.

In Table 3, we can see that rfPred has a higher index than the logistic regression model on the learning dataset in both cases.

Table 3: Partial Area Index calculated on the learning dataset

Models	rfPred (OOB*)	Logistic regression	CADD	Mutation Taster	SIFT	Polyphen 2
Sensitivity between 0,9 and 1	0,316	0,285	0,246	0,241	0,197	0,220
Specificity between 0,9 and 1	0,414	0,375	0,196	0,311	0,202	0,210

*OOB = Out of bag data

3.2 Model Validation on independent data

On the four validation datasets, each time rfPred is in par with the best performing predictors (Figure 2). The rfPred AUC is 0,90 on EXOVAR dataset (vs 0,84 for Polyphen2 and 0,86 for Mutation Taster), of 0,86 on COL7A1 (vs 0,85 for Polyphen2), of 0,95 on COL4A5 (vs 0,946 for SIFT). On the ClinVar-1KG validation set, rfPred is clearly more accurate than the logistic regression framework. Although the most reliable predictor seems to be Mutation Taster on the EXOVAR dataset, on the two specific gene-based validation datasets, SIFT and Polyphen2 give better results (Table 4). The strength of rfPred is that in all these datasets, it is the only one to remain among the most accurate ones.

If we consider now the partial AUC for high sensitivity or high specificity, the rfPred method is especially effective to detect disease causing variants with a minimum of false positives (high specificity). The logistic regression model outperforms the rfPred model for partial AUC with high sensitivity in two datasets from the four.

Table 4: Models comparison on the different validation datasets

Models	rfPred	Logistic regression	Mutation Taster	SIFT	Polyphen 2	CADD
Total AUC (EXOVAR)	0,90*	0,90	0,86	0,80	0,85	0,84
Partial AUC Index Sensitivity between 0,9 and 1 (EXOVAR)	0,46	0,50	0,41	0,25	0,37	0,39
Partial AUC Index Specificity between 0,9 and 1 (EXOVAR)	0,58	0,55	0,45	0,22	0,39	0,31
Total AUC (ClinVar-1KG)	0,91	0,82	NA	0,58	0,76	0,83
Partial AUC Index Sensitivity between 0,9 and 1 (ClinVar-1KG)	0,52	0,48	NA	0,02	0,14	0,41
Partial AUC Index Specificity between 0,9 and 1 (ClinVar-1KG)	0,52	0,35	NA	0,20	0,34	0,40
Total AUC (COL4A5)	0,95	0,95	0,92	0,95	0,94	0,87
Partial AUC Index Sensitivity between 0,9 and 1 (COL4A5)	0,72	0,72	0,53	0,75	0,70	0,66
Partial AUC Index Specificity between 0,9 and 1 (COL4A5)	0,73	0,71	0,74	0,62	0,63	0,13
Total AUC (COL7A1)	0,86	0,84	0,76	0,83	0,85	0,75
Partial AUC Index Sensitivity between 0,9 and 1 (COL7A1)	0,29	0,40	0,21	0,28	0,36	0,26
Partial AUC Index Specificity between 0,9 and 1 (COL7A1)	0,48	0,30	0,16	0,31	0,50	0,18

*Figures in bold are corresponding to the best result of the row

Fig 2: ROC curves on validation datasets for best classifiers

A: EXOVAR validation set, B: ClinVar - 1Kg validation set, C: COL4A5 validation set, D: COL7A1 validation set (LRT and PhyloP are not shown because their ROC curves are below the other ones)

4. Discussion and Conclusion

The protein function alteration prediction of a genetic SNP variant located on a coding DNA domain is a real challenge today. Many parameters should be taken into account, more or less bounded to each other, and the existing pathogenicity prediction methods give complementary information. The added value of combining several existing predictors is well established.

Although a logistic regression framework has already been proposed and seems to improve the accuracy of the five prediction scores, we have chosen a classification model based on a machine learning approach (random Forest) so that the method can take into account the nonlinear part of the link between variant pathogenicity and their prediction scores, and can model complex interactions between them. Such interactions would be complicated to define within a classical multiplicative model framework (Supplementary Data). The resulting rfPred model seems not only to be more accurate than the logistic regression framework already proposed and the CADD score for missense variants, but is also easily available to the scientific community.

rfPred is built with 5 000 trees, which was found to be the best compromise between complexity and accuracy on our training dataset; with 10 000 trees, the classification errors do not decrease and the forest stability does not increase anymore significantly (data not shown).

Concerning the learning dataset we have deliberately chosen to work with the neutral variants which have a frequency < 1% in the 1000 Genomes Project. Indeed, more frequent variants are easily excluded from

sets of possible causal variants in monogenic diseases without resorting to any statistical tool. According to us, the added-value of prediction methods for monogenic diseases appears precisely when a high number of variants remain potentially pathogenic even after filtering on the minor allele frequency in the general population. Our model is intentionally built on the more challenging dataset in term of classification; the implicit hypothesis is that the genetic variants with a frequency higher than 1% in the population are more often correctly classified as neutral by function alteration predictors. We have checked this hypothesis in comparing the rfPred scores distribution according to allele frequency of neutral variant (Supplementary data) and seen that the neutral variants with a MAF > 1% have globally lower rfPred scores than the rare neutral SNVs.

rfPred has been compared with other classification models coming from machine learning or statistical learning communities as Support Vector Machine (SVM) or boosting technique ²⁰. A recent example of such a tool is the CADD method ⁶, and rfPred seems really more performant on our different datasets. CADD offers the great advantage to provide scores for all the possible Single Nucleotide Variants of the human reference genome and not only for coding variants, but in the particular field of missense single nucleotide variations, it does not seem to be the most accurate one.

It could be interesting to add other prediction scores in our composite model, in particular those which have demonstrated a very good accuracy in particular contexts, like Mutpred ²⁷ or SNP&GO ²⁸. The recently releases of dbNSFP v2 introduce a few others predictors which could be used as well.

Another following step could be to integrate such a model in a more complex tool like ANNOVAR or VAAST 2.0 ²⁹ which allows finding candidate genes from phenotypically homogeneous exomes. VAAST 2.0 enables to join several approaches: the approach linked to the variant prioritization based on the amino-acid substitution (those which led us to develop rfPred), and the association analysis approach, based on the comparison between cases and controls. In the unified likelihood model of the tool, the variant prioritization is taken into account through an approach based on a conservation measurement PhastCons; a variant prioritization model like ours could further improve this tool, whatever its use in common diseases or in rare diseases.

Finally, to decrease the request time, rfPred scores have been pre calculated for each possible variation in the human exome and are available in tabix files downloadable at <http://www.sbim.fr/rfPred>. A R package downloadable on Bioconductor.org queries the data files stored directly on the server website, or locally downloaded. For a query of 200 variations scores, the request time is about 1s if the data files are stored locally on the computer (versus 6s if the data files are on the website). It is also possible to download the random forest model (in R.Data format) to compute the rfPred scores directly from the five LRT, SIFT, Polyphen2, PhyloP and MutationTaster scores.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

- 1 Ng PC, Henikoff S. Predicting the effects of amino acid substitutions on protein function. *Annu Rev Genomics Hum Genet* 2006; **7**: 61–80.
- 2 Thusberg J, Olatubosun A, Vihinen M. Performance of mutation pathogenicity prediction methods on missense variants. *Hum Mutat* 2011; **32**: 358–368.
- 3 Cooper GM, Shendure J. Needles in stacks of needles: finding disease-causal variants in a wealth of genomic data. *Nat Rev Genet* 2011; **12**: 628–640.
- 4 Liu X, Jian X, Boerwinkle E. dbNSFP: a lightweight database of human nonsynonymous SNPs and their functional predictions. *Hum Mutat* 2011; **32**: 894–899.
- 5 Li M-X, Gui H-S, Kwan JSH, Bao S-Y, Sham PC. A comprehensive framework for prioritizing variants in exome sequencing studies of Mendelian diseases. *Nucleic Acids Res* 2012; **40**: e53–e53.
- 6 Kircher M, Witten DM, Jain P, O’Roak BJ, Cooper GM, Shendure J. A general framework for estimating the relative pathogenicity of human genetic variants. *Nat Genet* 2014. doi:10.1038/ng.2892.
- 7 McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University (Baltimore, MD). Online Mendelian Inheritance in Man, OMIM®. 2012.<http://omim.org/> (accessed 12 May2014).

- 8 Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. *Nucleic Acids Res* 2010; **38**: e164.
- 9 Kumar P, Henikoff S, Ng PC. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. *Nat Protoc* 2009; **4**: 1073–1081.
- 10 Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P *et al.* A method and server for predicting damaging missense mutations. *Nat Methods* 2010; **7**: 248–249.
- 11 Schwarz JM, Rödelsperger C, Schuelke M, Seelow D. MutationTaster evaluates disease-causing potential of sequence alterations. *Nat Methods* 2010; **7**: 575–576.
- 12 Muse SV, Gaut BS. A likelihood approach for comparing synonymous and nonsynonymous nucleotide substitution rates, with application to the chloroplast genome. *Mol Biol Evol* 1994; **11**: 715–724.
- 13 Margulies EH, Cooper GM, Asimenos G, Thomas DJ, Dewey CN, Siepel A *et al.* Analyses of deep mammalian sequence alignments and constraint predictions for 1% of the human genome. *Genome Res* 2007; **17**: 760–774.
- 14 Pruitt KD, Harrow J, Harte RA, Wallin C, Diekhans M, Maglott DR *et al.* The consensus coding sequence (CCDS) project: Identifying a common protein-coding gene set for the human and mouse genomes. *Genome Res* 2009; **19**: 1316–1323.
- 15 1000 Genomes Project Consortium, Abecasis GR, Auton A, Brooks LD, DePristo MA, Durbin RM *et al.* An integrated map of genetic variation from 1,092 human genomes. *Nature* 2012; **491**: 56–65.
- 16 Li M-X, Kwan JSH, Bao S-Y, Yang W, Ho S-L, Song Y-Q *et al.* Predicting Mendelian Disease-Causing Non-Synonymous Single Nucleotide Variants in Exome Sequencing Studies. *PLoS Genet* 2013; **9**: e1003143.
- 17 Database of Single Nucleotide Polymorphisms (dbSNP). National Center for Biotechnology Information, National Library of Medicine. (dbSNP Build ID: 137). Bethesda (MD). <http://www.ncbi.nlm.nih.gov/SNP/>.
- 18 Stenson PD, Mort M, Ball EV, Howells K, Phillips AD, Thomas NS *et al.* The Human Gene Mutation Database: 2008 update. *Genome Med* 2009; **1**: 13.
- 19 Wertheim-Tysarowska K, Sobczyńska-Tomaszewska A, Kowalewski C, Skroński M, Świękowski G, Kutkowska-Każmierczak A *et al.* The COL7A1 mutation database. *Hum Mutat* 2012; **33**: 327–331.
- 20 Hastie T, Tibshirani R, Friedman J. *The elements of statistical learning*. Springer Series in Statistics. 2001.
- 21 Breiman. *RANDOM FORESTS: Machine Learning* - p5-32. 2001.
- 22 Breiman L. Manual On Setting Up, Using, And Understanding Random Forests V3.1. 2002. http://oz.berkeley.edu/users/breiman/Using_random_forests_V3.1.pdf (accessed 12 May2014).
- 23 Liu X, Jian X, Boerwinkle E. dbNSFP v2.0: a database of human non-synonymous SNVs and their functional predictions and annotations. *Hum Mutat* 2013; **34**: E2393–2402.
- 24 Marlow AJ, Fisher SE, Francks C, MacPhie IL, Cherny SS, Richardson AJ *et al.* Use of multivariate linkage analysis for dissection of a complex cognitive trait. *Am J Hum Genet* 2003; **72**: 561–570.
- 25 Crookston NL, Finley AO. yaImpute: An R package for kNN imputation. *J Stat Softw* 2008; **23**: 1–16.
- 26 Zhou X-H, Obuchowski NA, Mc Clish DK. *Statistical Methods in Diagnostic Medicine*. 2nd ed. Wiley.
- 27 Li B, Krishnan VG, Mort ME, Xin F, Kamati KK, Cooper DN *et al.* Automated inference of molecular mechanisms of disease from amino acid substitutions. *Bioinforma Oxf Engl* 2009; **25**: 2744–2750.
- 28 Calabrese R, Capriotti E, Fariselli P, Martelli PL, Casadio R. Functional annotations improve the predictive score of human disease-related mutations in proteins. *Hum Mutat* 2009; **30**: 1237–1244.
- 29 Hu H, Huff CD, Moore B, Flygare S, Reese MG, Yandell M. VAAST 2.0: Improved Variant Classification and Disease-Gene Identification Using a Conservation-Controlled Amino Acid Substitution Matrix: VAAST 2.0. *Genet Epidemiol* 2013; **37**: 622–634.