Evaluating the relevance of sequence conservation in the prediction of pathogenic missense variants

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Abstract
Evolutionary information is the primary tool for detecting functional conservation in nucleic acid and protein. This information has been extensively used to predict structure, interactions and functions in macromolecules. Pathogenicity prediction models rely on multiple sequence alignment information at different levels. However, most accurate genome-wide variant deleteriousness ranking algorithms consider different features to assess the impact of variants. Here, we analyze three different ways of extracting evolutionary information from sequence alignments in the context of pathogenicity predictions at DNA and protein levels. We showed that protein sequence-based information is slightly more informative in the annotation of Clinvar missense variants than those obtained at the DNA level. Furthermore, to achieve the performance of state-of-the-art methods, such as CADD and REVEL, the conservation of reference and variant, encoded as frequencies of reference/alternate alleles or wild-type/mutant residues, should be included. Our results on a large set of missense variants show that a basic method based on three input features derived from the protein sequence profile performs similarly to the CADD algorithm which uses hundreds of genomic features. As expected, our method results in ~3% lower area under the receiver-operating characteristic curve (AUC). When compared with an ensemble-based algorithm (REVEL). Nevertheless, the combination of predictions of multiple methods can help to identify more reliable predictions. These observations indicate that for missense variants, evolutionary information, when properly encoded, plays the primary role in ranking pathogenicity.

Introduction
High-throughput sequencing technologies have changed our daily research by rapidly accumulating genomic data and helping to profile patient genomes (MacArthur et al. 2014; Claussnitzer et al. 2020). These studies make variant interpretation a fundamental challenge in precision medicine (Fernald et al. 2011; Capriotti et al. 2012; McInnes et al. 2021). Missense variants by changing a single amino acid in a protein sequence can be neutral or induce loss of function.

In the last 2 decades, several methods have been developed to prioritize functional missense variants relying on protein sequence/structure information (Tennessen et al. 2012; Niroula and Vihinen 2016; Ancien et al. 2018; Petrosino et al. 2021) and the protein interaction networks (Rost et al. 2016; Capriotti et al. 2019; Ozturk and Carter 2021).

It is widely accepted that evolutionary information encoded in multiple sequence alignments of DNAs and proteins is a major resource for scoring variant pathogenicity. Several methods for scoring the nucleotide and amino acid conservation have been defined (Schneider 1997; Valdar 2002). Although there is no rigorous test for judging a conservation measure, in general, quantitative conservation measures are site-specific scores calculated from a vector representing the relative frequency of the amino acids or nucleotides in a given position of a multiple sequence alignment. Among the most commonly used scores are those calculating the Euclidean distance between two sets of amino acid frequencies (Valdar 2002) and Shannon’s information-theoretic entropy (Capra and Singh 2007). Such site-specific scores, which are important for identifying functionally conserved regions, do not explicitly depend on the pairs of wild-type and mutant nucleotides/amino acids observed in a specific mutation process. For scoring the pathogenicity of
a specific variation, we considered the most basic quantitative measures calculating the frequency of the wild-type and mutant nucleotides/residues in a given site.

This paper evaluates the relevance of this information for missense variant predictions by comparing simple scores and simple predictors with the widely used and well-performing Combined Annotation-Dependent Depletion (CADD) algorithm (Rentzsch et al. 2019) and REVEL (Ioannidis et al. 2016).

We computed the conservation scores on DNA (PhastCons100way and PhyloP100way), the frequencies of the reference and alternative alleles in the genome, and frequencies of the wild-type and mutant residues in protein multiple alignments. Our analysis showed that a machine-learning method trained on a few sequence conservation features at DNA or protein levels, achieves similar performance of a state-of-the-art algorithm. In this work, we compared the performance of CADD and REVEL with those reached by three different basic gradient boosting algorithms on a set of missense variants from the Clinvar database. Our result indicates that the evolutionary information provides the main features for scoring the pathogenicity of missense variants.

**Materials and methods**

**Datasets**

To evaluate the performance of different machine-learning methods for predicting the pathogenicity of missense variants, we collected two datasets from the Clinvar database (Landrum et al. 2020). For building the two datasets, we considered two versions of Clinvar released in June 2020 and August 2021, respectively. The first dataset (CommonClinvar) consists of the missense variants annotated as Pathogenic and Benign in both versions of the database while the second dataset (NewClinvar) collects the new missense variants reported in the last version of Clinvar since June 2020 (Fig. S1). The variants reported in the older version of Clinvar not confirmed in the last version were discarded. Thus, the CommonClinvar consists of 36,751 missense variants from 7582 proteins 53.5% of which are annotated as Benign and the remaining ones (46.5%) as Pathogenic. NewClinvar, which includes only the newly annotated variants, is composed of 5172 from 1855 proteins 43.4% of which are reported as Benign and 56.6% as Pathogenic. The composition of the two datasets is summarized in Table S1. Both CommonClinvar and NewClinvar datasets are available as supplementary files.

**Conservation features**

In this work, we analyzed the performance in the prediction of pathogenic variants using three basic methods based on conservation features. Each method considers only three input features, which are described as follows.

As a baseline, we implemented a method considering two site-specific conservation scores (PPScore) calculated on a genome level multiple sequence alignment and made available through the UCSC genome browser (Kent et al. 2002). The conservation scores used in the first method are calculated by PhastCons (Siepel et al. 2005) and PhyloP (Pollard et al. 2010) algorithms.

In the second method (DNAProf), the frequencies of the reference and alternative alleles in the mutated site are calculated for each variant from the multiz100way multiple sequence alignment. The PhastCons100 and PhyloP100way scores as well as the multiz100way alignments for the hg38 human reference genome are available at https://hgdownload.cse.ucsc.edu/goldenpath/hg38/.

In the third method (ProtProf), we calculated the frequencies of the wild-type and mutant residues in the mutated sites for each mutation. These frequencies are derived from the BLAST (Altschul et al. 1997) search alignments against the UniRef90 database (Suzek et al. 2007) released in June 2020. For the BLAST search, we used an e-value cutoff of $10^{-9}$ as suggested in previous works (Capriotti et al. 2006, 2013, 2017; Calabrese et al. 2009). By definition, the E-value represents the number of expected hits found by chance and depends on the number of sequences in the database. In our case, selecting an E-value threshold of $10^{-9}$ for a BLAST search on UniRef90, which contains $\sim 10^8$ sequences, ensures that less than one random hit can be found by chance from our search.

In summary, for each mutated loci, we considered the PhastCons100way (PC) and PhyloP100way (PP), the frequencies of the reference ($f_{\text{ref}}$) and alternative ($f_{\text{alt}}$) alleles from the multiz100way multiple sequence alignment, and the total number of aligned genomic sequences ($N_g$). For the protein-based method, the protein sequence profile was obtained considering the sequences returned by BLAST. For each mutated site, we calculated the frequencies of wild-type ($f_{\text{wt}}$) and mutant ($f_{\text{mut}}$) residues and the number of aligned proteins ($N_p$). The nucleotide and amino acid frequencies are calculated as follows:

$$f(x_i) = \frac{n(x_i)}{\sum_{i=1}^{k} n(x_i)},$$

where $n(x_i)$ is the number of the nucleotide or amino acid $x_i$ in the sequence alignment and $k$ is equal to 5 (including the generic nucleotide $N$) and 20 for DNA and protein sequences, respectively. $N_g$ and $N_p$ represent the
denominators of the equation above for DNA and protein sequences, respectively.

**Machine-learning algorithms**

Using the eight features described above, we develop three binary classifiers (PPScore, DNAProf, ProtProf) using the following groups of three features:

- **PPScore** PhastCons100way (PC) and PhyloP100way (PP) scores, and number aligned genomic sequences ($N_g$) in multiz100way
- **DNAProf** Frequencies of the reference ($f_{\text{ref}}$) and alternative ($f_{\text{alt}}$) alleles, and number aligned genomic sequences in multiz100way ($N_g$).
- **ProtProf** Frequencies of the wild-type ($f_{\text{wt}}$) and mutant ($f_{\text{mut}}$) residues, and number aligned protein sequences ($N_p$) from a BLAST search on UniRef90.

For each group of features defined above, we developed a binary classifier based on the gradient boosting algorithm as implemented in the scikit-learn package (Pedregosa et al. 2011). The proposed groups of features are summarized in Table 1.

**Training and testing procedure**

We first evaluated the performance of each method on CommonClinvar using a tenfold cross-validation procedure for a fair evaluation of the proposed method performance. To reduce at the minimum the possible overfitting, we mapped each missense variant on the relative protein sequence and we clustered all the sequences using the blastclust algorithm (https://ftp.ncbi.nih.gov/blast/documents/blastclust.html) with a sequence identity threshold of 25% and a coverage of 50%. Using the clustering based on sequence similarity, we perform a tenfold cross-validation procedure keeping all the variants belonging to the same cluster in the same sub-set. A second test is performed considering the NewClinvar dataset. In this case, the impact of the variants of a given protein is predicted excluding from the training set (CommonClinvar dataset) all the variants belonging to proteins of the same cluster. We extracted a balanced set of Pathogenic and Benign variants from CommonClinvar and NewClinvar dataset for each test, randomly downscaling the most abundant class. The reported scoring measures for all the methods are averaged over ten randomly selected sets.

**Benchmarking and performance measures**

To characterize the prediction power of the main features described in this work, for each of them, we developed a single feature binary classifier based on a single threshold. For each feature, the classification threshold is optimized on the CommonClinvar dataset maximizing both the true-positive and true-negative rates. The optimized threshold is tested in the classification of the NewClinvar variant dataset.

Finally, the performances of all the binary classifiers described above are compared with those achieved by the CADD (Rentzsch et al. 2019) and REVEL (Ioannidis et al. 2016) algorithms. The optimized raw score threshold for the classification of CADD output was calculated on the CommonClinvar dataset as binary classifier. The performances of the methods are scored considering two subsets of the NewClinvar dataset including the consensus predictions (Consensus) and those with at least one predictor in disagreement with the remaining ones (NotConsensus).

All the measures considered for scoring the performance of the methods are defined in Supplementary Materials.

**Results**

**Feature analysis and single feature classification**

In the first part of our work, we analyzed the distributions of the main features used for the classification task. We focused on the six conservation features (PC, PP, $f_{\text{ref}}$, $f_{\text{alt}}$, $f_{\text{wt}}$, $f_{\text{mut}}$) comparing their distributions for the subsets of Pathogenic and Benign variants. The average, median and standard deviation of such distributions are reported in Table S2. As observed in previous works (Kircher et al. 2014; Capriotti and Fariselli 2017), the distribution of the PhyloP100way score (PP) in mutated loci associated with Pathogenic and Benign variants are significantly different (Fig. 1). Indeed, the two distributions show median values of 7.5 and 1.5, respectively, with a Kolmogorov–Smirnov distance ($D_{KS}$) of 0.57 (Fig. 1B and Table S2). This distance is greater than the $D_{KS}$ observed for the PhastCons100way score (PC).

A higher difference between the distributions of the conservation scores for the subset of Pathogenic and Benign variants is observed when the frequencies in sequence profile from genomic and proteins are considered. The most remarkable differences are generally detected when comparing the distributions of the frequency of the alternative allele ($f_{\text{alt}}$) and the mutant residue ($f_{\text{mut}}$) for which the $D_{KS}$ is ~0.60. Analyzing the frequencies of the reference allele ($f_{\text{ref}}$) and wild-type residue ($f_{\text{wt}}$) their $D_{KS}$ is 0.58 and 0.55.

| Table 1 | Three groups of features used for the development of the binary classifiers |
|---------|--------------------------|
| **Group** | **Features** |
| PPScore | PC PP $N_g$ |
| DNAProf | $f_{\text{ref}}$ $f_{\text{alt}}$ $N_g$ |
| ProtProf | $f_{\text{wt}}$ $f_{\text{mut}}$ $N_p$ |
respectively (Table S2). The distributions of the four types of frequencies (\(f_{\text{ref}}\), \(f_{\text{alt}}\), \(f_{\text{wt}}\), and \(f_{\text{mut}}\)) for the subsets of Pathogenic and Benign variants are plotted in Fig. 2.

This observation agrees with the results obtained in the prediction of Pathogenic variants using a classification threshold on a single feature. The classification threshold is optimized on the CommonClinvar dataset maximizing both the true-positive and -negative rates (Table S3). Applying the optimized thresholds on the prediction of the variants in the NewClinvar dataset, we found that a simple classifier...
based on the frequency of the mutant residue extracted from a protein sequence profile achieve 81% overall accuracy (Q2), 0.63 Matthews correlation coefficient (MCC) and an Area Under the Receiver-Operating Characteristic Curve (AUC) of 0.86 (Table 2).

According to the previous observation, the PhastCons100way score (PC) is the least discriminating feature. When using the optimized threshold on the classification of the NewClinvar variants, the method based on a single PC threshold achieves 74% overall accuracy, 0.49 MCC and 0.75 AUC (Table 2). Slightly lower performances are obtained when the frequencies of the reference allele and the wild-type residue in the sequence profile are considered. In this case, the method based on a single \( f_{\text{ref}} \) threshold results in 78% Q2, 0.56 MCC and 0.84 AUC. These results can also be observed plotting the Receiving-Operating Characteristic (ROC) and Precision-Recall (PR) curves reported in Fig. S2.

Assessment of the machine-learning methods

Starting from the previous observations, we developed three machine-learning approaches based on the different groups of conservation features. The PPScore method is based on the PhastCons100way, PhyloP100way scores representing unique conservation measures not describing the type of nucleotides observed in the mutated loci. The other two methods consider the frequencies of the nucleotides or residues in the original and new sequences that correspond to \( f_{\text{ref}}, f_{\text{wt}} \) and \( f_{\text{mut}} \) for DNAPref and ProtProf, respectively. To these groups of measures, we added a third feature representing the total number of sequences aligned in the mutated loci (\( N_g, N_p \)). Although these values are not related to the conservation, they are considered as features for differentiating cases of mutated loci aligned with low and high number of sequences. We implemented three machine-learning methods for predicting Pathogenic variants based on the gradient boosting algorithm with these groups of features. First, the performance of these methods is tested with a tenfold cross-validation procedure on the CommonClinvar dataset. To avoid possible overfitting, we clustered all the proteins based on the sequence identity and grouped all their variants in a unique subset. The average performance of PPScore, DNAPref and ProtProf on a balanced set of Pathogenic and Benign variants is reported in Table S4. The results show that among the three methods ProtProf, which is based on protein sequence profile, achieved the highest performance reaching 83% overall accuracy (Q2), 0.67 Matthews correlation coefficient and 0.91 Area Under the Receiver-Operating Characteristic Curve (AUC). PPScore which is based on PhastCons100way, PhyloP100way show the lowest performance resulting in ~4% lower AUC and ~9% lower MCC. An intermediate level of performance is achieved by DNAPref which results in ~2% lower AUC and ~3% lower MCC with respect to ProtProf. Similar results are obtained when assessing the performance of the three methods on the NewClinvar dataset. In addition, in this case, we predicted the impact of each variant removing from the training set all the variants in the CommonClinvar training set belonging to the same cluster of proteins. The performance of PPScore, DNAPref and ProtProf on a balanced set of variants from the NewClinvar dataset is summarized in Table 3. For scoring the contribution of \( N_g \) and \( N_p \) to the predictions of the three methods, we removed such features from the input and compared their performances. The results reported in Table S5 show that the methods improve their performance by ~1% for MCC and AUC indices considering \( N_g \) and \( N_p \) in the input features.

Comparison with CADD and REVEL algorithms

In the final part of our analysis, we compared the performance of our simple gradient boosting-based algorithms with those obtained with CADD (Rentzsch et al. 2019) and REVEL (Ioannidis et al. 2016). REVEL (Rare Exome Variant Ensemble Learner) is an ensemble method for predicting the pathogenicity of missense variants on the basis of 13 individual tools. When tested on independent test sets, REVEL shows the best overall performance as compared to any of the individual tools and 7 previously developed ensemble methods. CADD is one of the most accurate and

| Feature | Threshold | Q2 | TNR | NPV | TPR | PPV | MCC | F1 | AUC | AUP |
|---------|-----------|----|-----|-----|-----|-----|-----|----|-----|-----|
| PC      | 1.000     | 0.737 | 0.611 | 0.816 | 0.862 | 0.689 | 0.489 | 0.766 | 0.755 | 0.815 |
| PP      | 4.704     | 0.769 | 0.796 | 0.756 | 0.743 | 0.784 | 0.539 | 0.763 | 0.841 | 0.828 |
| \( f_{\text{ref}} \) | 0.977 | 0.779 | 0.815 | 0.760 | 0.742 | 0.801 | 0.559 | 0.770 | 0.836 | 0.843 |
| \( f_{\text{wt}} \) | 0.000 | 0.794 | 0.750 | 0.821 | 0.837 | 0.770 | 0.589 | 0.802 | 0.828 | 0.863 |
| \( f_{\text{mut}} \) | 0.702 | 0.769 | 0.806 | 0.750 | 0.731 | 0.791 | 0.539 | 0.759 | 0.844 | 0.836 |
| \( f_{\text{mut}} \) | 0.005 | 0.815 | 0.819 | 0.812 | 0.810 | 0.817 | 0.629 | 0.814 | 0.857 | 0.856 |

Q2 overall accuracy, TNR true-negative rate, NPV negative predicted value, TPR true-positive rate, PPV positive predicted value, MCC Matthews Correlation Coefficient, F1 harmonic mean of precision and sensitivity, AUC area under the receiver operator characteristic curve, AUP area under the precision recall curve. All the performance measures are defined in Supplementary Materials.
Table 3 Testing prediction on the NewClinvar variant dataset

| Method    | Q2   | TNR  | NPV  | TPR  | PPV  | MCC  | F1   | AUC  | AUP  |
|-----------|------|------|------|------|------|------|------|------|------|
| CADD      | 0.844| 0.821| 0.860| 0.867| 0.829| 0.688| 0.847| 0.911| 0.905|
| REVEL     | 0.871| 0.918| 0.843| 0.825| 0.909| 0.747| 0.865| 0.945| 0.942|
| ProtProf  | 0.831| 0.865| 0.809| 0.796| 0.855| 0.662| 0.824| 0.910| 0.905|
| DNAProf   | 0.812| 0.780| 0.834| 0.845| 0.794| 0.626| 0.818| 0.881| 0.873|
| PPScore   | 0.771| 0.776| 0.769| 0.767| 0.774| 0.543| 0.770| 0.855| 0.846|

All the performance measures are defined in Supplementary Materials. For CADD, a raw score classification threshold of 3.1 was considered.

Q2 overall accuracy, TNR true-negative rate, NPV negative predicted value, TPR true-positive rate, PPV positive predicted value, MCC Matthews Correlation Coefficient, F1 harmonic mean of precision and sensitivity, AUC area under the receiver operator characteristic curve, AUP area under the precision recall curve. All the performance measures are defined in Supplementary Materials.

popular methods for predicting Pathogenic variants in coding and non-coding regions (Benevenuta et al. 2021). This method, which is based on more than hundreds of genomic features, was trained on more than 30 million variants. To use CADD as a binary classifier, we considered the raw output of the program and we selected the threshold that maximizes the true-positive and -negative rates on the CommonClinvar dataset. The performance of CADD at the optimal raw score classification threshold of 3.1 is reported in Table S4. This threshold was used for the classification of the variants in the NewClinvar dataset. The performances of CADD and REVEL on the NewClinvar dataset are summarized in Table 3. This analysis shows that CADD and ProtProf algorithms result in a similar performance in the classification of Pathogenic missense variants in terms of Area Under the Receiver-Operating Characteristic (AUC) and Precision-Recall (AUP) curves on both CommonClinvar and NewClinvar datasets. As expected, REVEL outperforms ProtProf and CADD reaching ~3% higher overall accuracy (Q2) and AUC. We can also observe that DNAProf which is based on the sequence profile extracted from the multiz100way sequence alignments results only in ~3% lower AUC and AUP. The Receiver-Operating Characteristic and Precision-Recall curves for CADD, REVEL and the three methods presented in this manuscript are plotted in Fig. 3.

Analysis and comparison of the predictions

To better understand the difference among the presented methods, we compared the predictions of three gradient boosting-based algorithms (PPScore, DNAProf and ProtProf). For performing this analysis, we defined two subsets of the NewClinvar dataset: the Consensus subset where the three predictions agree and the NotConsensus subset where the predictions differ. When performing such comparison, we observe that predictions overlap in ~73% of the cases in

![Fig. 3 Receiver-Operating Characteristic (A) and Precision-Recall (B) curves for the different gradient boosting algorithms (PPScore, DNAProf, ProtProf), CADD and REVEL on the NewClinvar dataset](image-url)
the *NewClinvar* dataset (Fig. 4A), while in the remaining 23% a single predictor differs from the other.

*ProtProf* and *DNAProf* provide the highest prediction similarity, agreeing in 88% of the cases with a correlation of 0.75 (Fig. 4C). In terms of performance, when focusing on the *Consensus* subset, the performance of *ProtProf* reaches 0.91 AUC and 0.81 Matthews correlation, while on the remaining subset, all the methods show an AUC < 0.65 and *PPScore* results on AUC < 0.5 (Table 4). The decrease in the performance can be explained by comparing the distribution of the frequencies of the wild-type and mutant residues on the *Consensus* and *NotConsensus* subsets. Indeed, the

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**Table 4** Performance of *PPScore*, *DNAProf* and *ProtProf* on two subsets of the *NewClinvar* dataset

| Method   | Subset          | Q2  | TNR  | NPV  | TPR  | PPV  | MCC  | F1   | AUC  | AUP  |
|----------|-----------------|-----|------|------|------|------|------|------|------|------|
| *ProtProf* | *Consensus*    | 0.905 | 0.907 | 0.909 | 0.904 | 0.901 | 0.810 | 0.902 | 0.905 | 0.929 |
| *ProtProf* | *NotConsensus* | 0.633 | 0.740 | 0.577 | 0.542 | 0.712 | 0.286 | 0.615 | 0.641 | 0.735 |
| *DNAProf* | *NotConsensus* | 0.567 | 0.402 | 0.536 | 0.706 | 0.583 | 0.113 | 0.639 | 0.554 | 0.665 |
| *PPScore* | *NotConsensus* | 0.418 | 0.385 | 0.369 | 0.445 | 0.461 | −0.170 | 0.453 | 0.415 | 0.561 |

*Consensus* Subset of *NewClinvar* (72.6%) for which the predictions of the three methods are in agreement, *NotConsensus* subset of *NewClinvar* (27.4%) for which one method differs from the remaining two

Q2 overall accuracy, TNR true-negative rate, NPV negative predicted value, TPR true-positive rate, PPV positive predicted value, MCC Matthews Correlation Coefficient, F1 harmonic mean of precision and sensitivity, AUC area under the receiver operator characteristic curve, AUP area under the precision recall curve. All the performance measures are defined in Supplementary Materials.

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**Fig. 4** Overlap of the predictions of the different methods on the *NewClinvar* dataset. A Venn diagram of the prediction of the 3 gradient boosting algorithms (*PPScore*, *DNAProf*, *ProtProf*) and B Venn diagram of the prediction of *ProtProf*, CADD and REVEL. C Pairwise overlap of the prediction of the 3 gradient boosting algorithms and D Pairwise overlap of the prediction of the *ProtProf*, CADD and REVEL. The numbers above the diagonal represent the fraction of common predictions while those below the diagonal are the correlations between the predictions.
Kolmogorov–Smirnov distance (DKS) of the distributions of fwt and fmut decrease by ~50% when considering the NotConsensus subset (Fig. 5).

A similar analysis is performed comparing the predictions of ProtProf, CADD and REVEL. Here, the predictions of the three algorithms overlap in ~75% of the cases (Fig. 4B) with an average of ~83% of common predictions and a correlation of ~0.68 for pairwise comparison (Fig. 4D). The performance of the methods on the Consensus subsets achieves ~95% in terms of overall accuracy and ~0.95 AUC, while for the NotConsensus subset, the performance of CADD and ProtProf are similar to those of a random predictor. REVEL shows the best performance on this subset of the NewClinvar dataset, achieving 67% Q2 and 0.68 AUC (Table 5).

The analysis of the distributions of $f_{wt}$ and $f_{mut}$ on shows a $D_{KS} > 0.70$ for the Consensus subset, while for the NotConsensus it drops below 0.2 with both the distributions of the fwt and fmut for Benign and Pathogenic variants strongly overlapping (Fig. 6).

**Conclusion and discussion**

Here, we analyzed different evolutionary information encodings for missense variant pathogenicity predictions. We compared the encoding at DNA and protein levels, where different multiple alignments techniques apply. The multiple sequence alignment includes a larger number of proteins and more remote homologs for many genes than which represents the maximum distance between the distributions of the frequencies for Pathogenic and Benign variants is reported. Average and standard deviations of the distributions are reported in Table S6.

![Fig. 5](image)

**Table 5** Performance of REVEL, CADD and ProtProf on two subsets of the NewClinvar dataset

| Method  | Subset          | Q2   | TNR  | NPV  | TPR  | PPV  | MCC  | F1   | AUC  | AUP  |
|---------|-----------------|------|------|------|------|------|------|------|------|------|
| ProtProf| Consensus       | 0.945| 0.954| 0.941| 0.935| 0.950| 0.890| 0.943| 0.945| 0.959|
| ProtProf| NotConsensus    | 0.479| 0.539| 0.429| 0.431| 0.541| −0.029| 0.480| 0.485| 0.616|
| REVEL   | NotConsensus    | 0.662| 0.777| 0.589| 0.570| 0.763| 0.350| 0.653| 0.674| 0.760|
| CADD    | NotConsensus    | 0.531| 0.337| 0.458| 0.684| 0.565| 0.022| 0.619| 0.510| 0.628|

Consensus: Subset of NewClinvar (75.5%) for which the predictions of the three methods are in agreement. NotConsensus: subset of NewClinvar (24.5%) for which one method differs from the remaining two

Q2 overall accuracy, TNR true-negative rate, NPV negative predicted value, TPR true-positive rate, PPV positive predicted value, MCC Matthews Correlation Coefficient, F1 harmonic mean of precision and sensitivity, AUC area under the receiver operator characteristic curve, AUP area under the precision recall curve. All the performance measures are defined in Supplementary Materials.
pre-calculated genome alignments from the UCSC genome browser. This condition can be the reason why the performance of a method trained using the protein-based information is slightly better. Thus, at least for the missense variants, an input based on evolutionary information of the wild-type and mutated residue performs better than evolutionary measures based on DNA sequence alignment in the prediction of pathogenic variants. Indeed, on the ~27% of the variants on which the predictions of our gradient boosting algorithms disagree, ProtProf reaches 6% high overall accuracy and 9% higher AUC with respect to DNAProf which rely on pre-calculated DNA sequence alignment from UCSC.

With these simple inputs based on evolutionary information, a machine-learning method can perform comparably to CADD, which uses more sophisticated inputs. When compared with an ensemble-based approach (RAVEL), our basic method (ProtProf) results in ~3% lower overall accuracy and AUC. Nevertheless, our analysis based on the comparison of the predictions of different methods allows the identification of a more reliable subset of predictions on which ProtProf reaches an overall accuracy above 90% and AUC > 0.9.

Recently, it has been suggested that protein positions have a significant role and can act as Neutral, Toggle or Rheostat (Miller et al. 2019). Here, we indicate an alternative view of protein positions that can be seen as a non-linear combination of the frequencies of wild-type/mutant residues at protein level or reference/alternative allele at DNA level. The results of our analysis suggest that the performances of new and more sophisticated machine-learning algorithms should always be compared with those achieved by simple conservation-based methods. As recently proposed (Walsh et al. 2021), the design of such benchmark tests should consider the adoption of specific guidelines for avoiding bias in the training and testing sets. This procedure is important to exclude overfitting on the context-dependent features (Grimm et al. 2015) and identify new important features for improving the performance of variant scoring algorithms.

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**Availability of data and material** Additional information is available in Supplementary Materials. The CommonClinvar and NewClinvar datasets are provided as supplementary files and available at https://doi.org/10.5281/zenodo.5722031.
Declarations

Conflict of interest None.

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