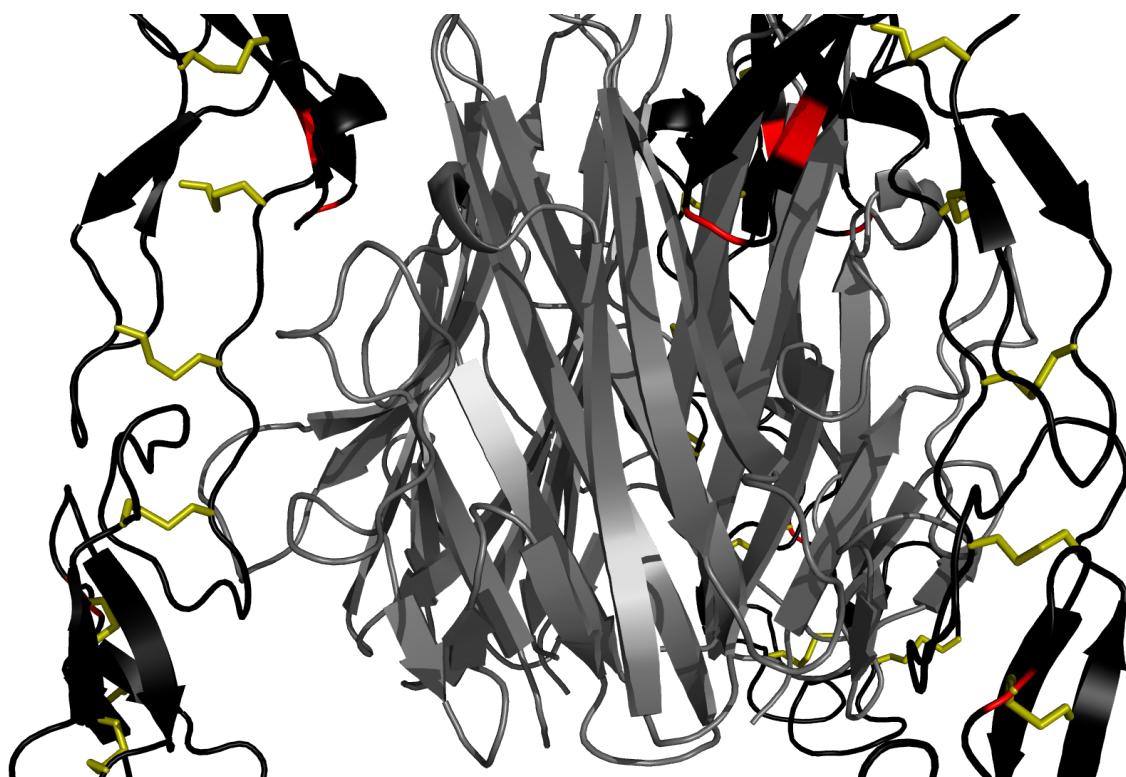


Protein structure modeling for variant pathogenicity prediction

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Protein structure modeling for variant pathogenicity prediction
Model of TNF β wild type structures with mutations that will be mutated highlighted in red.
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Abstract

Around 1 in 17 people is affected by one of 7,000 known rare diseases. Most of these patients do not receive a diagnosis, which means they remain in uncertainty without a prognosis, are unable to join specific patient support groups, and do not receive the most appropriate treatment. Next-generation sequencing (NGS) of DNA promises to establish a molecular diagnosis and help these patients but many challenges still stand in the way of maximum success. Recent years have seen great advances in computational tools that quickly reduce the amount of DNA variants to be interpreted by a human expert for potentially pathogenic effects. But, the current tools that rely on features such as evolutionary conservation, annotation of regulatory genomics elements and structural DNA features have been already optimized over many years and significant improvements are not expected. Here we tried to introduce structural features of proteins into diagnostics based on the methods used by VIPUR. Through the difficulties of protein modeling and experts knowledge it was discovered that methods used by VIPUR are not features that can help in diagnosis with machine learning. Structural data of proteins is often incomplete and is highly dependent on experimentally determined structures which is expensive. The methods that VIPUR uses to standardize protein structures for machine learning removes the context and treats them like they are in a vacuum. To gain a more realistic view on structural information we chose to use the web service HOPE and to develop a method to gain insight in the structural features of a protein and several variants, with we try to achieve the collection of structural information for diagnosis.

Acknowledgment

This graduation project has taken place at Universitary Medical Center Groningen (UMCG) at the Department of Genetics within the genomics coordination center (GCC), this report has been written for the GCC to gain insight into structural data to improve the GAVIN variant predictor. I want to thank Joeri van der Velde for supervising me during this project and helping me with writing this report. I also would like to thank Benjamin Kant and Marielle van Gijn for helping me decide to focus on assessing tumor necrosis factor associated receptor-associated periodic syndrome (TRAPS). And I especially would like to thank Tsjerk Wassenaar for informing us about the function and structure of TNFRSF1A, giving us the appropriate protein structures to work with and steering this project into a meaningful direction.

Abbreviations

3D Three Dimensional
ACCP Solvent Accessible Surface Area
API Application Programming Interface
Bash Bourne Again Shell
CPU Central Processing Unit
CSV Comma Separated Values
DNA Deoxyribonucleic Acid
FADD Fas Associated Death Domain protein
FEM Fixed End Move
FHF Familial Hibernian Fever
GAVIN Gene-Aware Variant INterpretation
GCC Genomic Coordination Center
GRCh/hg Genome Reference Consortium Human Human Genome
HOPE Have yOur Protein Explained
LOMETS Local Meta-threading Server
MD Molecular Dynamics
MPI Message Parsing Interface
NCBI National Center for Biotechnology Information
NF- κ B Nuclear Factor kappa-light-chain-enhancer of activated B cells
OpenGL Open Graphics Library
OS Operating System
OSF Open Science Framework
PDB Protein Data Bank
PM Pivot Movement
PSI-BLAST Position Specific Iterative BLAST
PSSM Position Specific Scoring Matrix
RCSB Research Collaboratory for Structural Bioinformatics
REU Rosetta Energy Unit
RNA Ribonucleic acid
RMSD Root Mean Square Deviation
SASA Solvent Accessible Surface Area
SCOP The Structural Classification of Proteins
SLURM Simple Linux Utility for Resource Management
SODD Silencer of Death Domain
SPVAA Simple Protein Variant Analysis Approach
TNF Tumor Necrosis Factor
TNF α Tumor Necrosis Factor Alpha
TNF α Tumor Necrosis Factor Alpha
TNF β Tumor Necrosis Factor Beta
TNFB Tumor Necrosis Factor Beta
TNFR1 Tumor Necrosis Factor Receptor Superfamily Member 1A TNFRSF1A Tumor Necrosis Factor Receptor Superfamily Member 1A
TRADD Tumor Necrosis Factor Receptor type 1-Associated DEATH Domain protein
TRAPS Tumor necrosis factor associated Receptor-Associated Periodic Syndrome
VIPUR Variant Interpretation Using Rosetta
VTS VIPUR Training Set

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1 Introduction

Around 1 in 17 people is affected by one of 7,000 known rare diseases. Most of these patients do not receive a diagnosis, which means they remain in uncertainty without a prognosis, are unable to join specific patient support groups, and do not receive the most appropriate treatment. Next-generation sequencing (NGS) of DNA promises to establish a molecular diagnosis and help these patients but many challenges still stand in the way of maximum success. Recent years have seen great advances in computational tools that quickly reduce the amount of DNA variants to be interpreted by a human expert for potentially pathogenic effects [1]. Although algorithms can now safely remove around 95% of the harmless variants, this still leaves hundreds of variants to be investigated for a whole-exome sequenced patient, which is far too many for a quick and clear diagnosis. Current tools to predict variant pathogenicity rely on features such as evolutionary conservation, annotation of regulatory genomics elements or structural DNA features. These tools have already been optimized over many years and further significant improvements are not expected. Therefore there is still a great need for even more powerful variant prioritization tools. A refreshing alternative was presented by variant interpretation using Rosetta (VIPUR) [2] which shows the potential of structural modeling of proteins to predict the actual effect of a specific variant on the function of a protein. However the publication did not result in a piece of high quality software that is usable for routine diagnostics. Therefore we want to test if the structural information used by VIPUR is useful to diagnostics with the difficult to assess gene tumor necrosis factor receptor 1 alpha (TNFRSF1a) to see if the prediction has any meaning. To test this we use two validated mutations of TNFRSF1A and one provisional mutation in combination with the tool have your protein explained (HOPE) [3] and a self developed method Simple Protein Variant Analyses Approach (SPVAA). Through testing these methods we will explore the potential pitfalls of protein modeling and discover if it is possible to add structural information to routine diagnostics.

2 Variant prediction in genome diagnostics and the addition of protein modeling

2.1 Mutations and its effects in the central dogma of molecular biology

Within the human genome mutations occur continuously by internal and external factors that: insert, remove, substitute or alter the reading frame in a nucleotide sequence. Mutations are not without consequences and can be protective [4], benign or harmful by altering the deoxyribonucleic acid (DNA) order. From a sequence of DNA genes are transcribed into ribonucleic acid (RNA) which can work as machinery or translates into an amino acid sequence to form a protein. Mutations outside a gene could lead to lowered or heightened transcription of a protein, when a mutation resides inside a gene it could lead to proteins that are unstable during or after formation, perform less optimal or are not functional [5–7].

2.2 A general concept of structural levels within proteins and the effect of mutations

The formation of protein structures is classified in different levels, distinctions are made based on bindings and structures that arise with the interaction of bonds. The order in which amino acids appear in a sequence is called the primary structure, in this level amino acids are only bound to each other by peptide bonds. Within a primary structure amino acids can form new peptide bonds between the N-terminus and C-terminus of an amino acid, with these bonds 3D structures are made called α -helices and β -sheets that together make up the secondary structure. The tertiary structure gives further rise to the 3D shape of a polypeptide by making disulfide bridges, ion and hydrogen -bonds, hydrophobic and hydrophilic -interactions between amino acids By combining multiple tertiary structures the quaternary structure of a protein can be formed out of the mentioned bonds, bridges and interactions [8, 9].

Mutations within proteins can have different effects to protein structures, often single missense mutations often have minimal effect on the backbone of a protein [10, 11] but can result in destabilization of the structure when assembled or can disrupt the active site. Frameshift mutations on the other hand can cause large differences in the primary structure and have therefore a higher chance of an altered sequence that leads to deformation or stop codon introduction [12].

2.3 Addition of structural data to diagnosis and treatment in healthcare

Acquiring information about DNA sequences depends on sequencing, which became cheaper over the years [13], and found its use in diagnosing patients within the healthcare sector [1]. From the collected data by genome sequencing experiments most of the analysis is handled in-silico due to the quantities of data that are produced [1]. Proteins often find their use in diagnosing diseases experimentally [9, 14], however in-silico it is often limited to information about conservation in the amino acid sequence [15]. Yet, the 3D shape of proteins defines their function [16] and by assessing structures it can become possible to determine changes in function that are caused by mutations that might not be discoverable through conservation and are therefore unclassifiable. Another advantage of structural information is that it becomes possible to develop treatment with diagnostic information for diseases that are caused by mutations [17]. With experimental methods such as X-ray crystallography and nuclear magnetic resonance (NMR) more than 158000 structures [18] have been completely revealed, however it is only a tiny fraction of the potential possible proteins [19] (especially without the inclusion of all folds). Making 3D structures is currently not common for diagnosis because it is relative expensive and is difficult to perform, some structures contain flexible regions which makes it hard to determine the exact position of some atoms and can cause information loss about the structure [20, 21].

2.4 Protein modeling techniques

An alternative approach for determining structures, compared to experimental methods, is protein modeling wherein structures are generated computationally. One of the benefits from making a structure in such a way is that laws of physics do not hinder the collection of structural information, but the lack of physics is also its weakness since the structures tend to be less accurate representations of proteins.

With the method homology modeling the amino acid sequences of proteins are aligned to sequences of experimental determined structures, based on these alignments a template is formed whereon structural fragments are built. It is not recommended to use this strategy if the sequence identity is less than 20% since there might not be any structural relation [11]. Another approach is protein threading which relies on the observation of folds in previously determined experimental structures, with the occurrence of specific folds a probability is predicted that determines how a protein folds in a specific manner.

Strategies are continuously being developed and improved to determine unknown structures, but all have partially similar guidelines wherein the avoidance of steric hindrance [22] and low energies, determined by scoring systems [23], are important. From the computer generated models many are less accurate than those experimentally determined structures, yet computational models could potentially gain the upper hand in solving membrane proteins[24].

2.5 A theoretical large scale implementation of structural protein variant assessment

With the wide spectrum of potential different proteins it can be difficult to produce any form of universal protein assessment tool, that is able to determine if a mutation is harmful or not based on structural information. However a first step to solve such a complex problem would be by determining the correct approach. In this case it is assumed that a machine learning would be the best method for detecting patterns in structures and classifying the effect of structural changes, because it has the ability to learn from structural mutations currently available and develop new insights [25].

Since the problem is so complex it should be divided into smaller more feasible problems, beginning by separating the different protein classes, which for example can be done according to The Structural Classification of Proteins database (SCOP) [26]. A first discrimination between the proteins could be made based on protein type/fold class (membrane, globular, fibrous and disordered -proteins) because these differences already predetermine some functions and locations for certain proteins in a cell [27–30]. After formation of these classes each should have its own machine learning method applied so their features can be analyzed within context of where and how they function. The next set of discriminators is highly dependent on the variations in classes, but all have features in the end describing bonds, interactions and movement of complexes in protein structures. When for each of the main classes methods have been developed a meta classifier determines which method should be applied to determine the effect of a mutation.

2.6 Monte Carlo method

There are complex problems in a variety of research fields which could take up years or even centuries to compute with simple deterministic methods. For some problems there is an algorithmic solution which makes it possible to cut down computation time significantly, but when no deterministic algorithm is available to speed up the process an empirical probabilistic method might be able to approximate the desired results.

With the Monte Carlo method random samples are taken from the parameter space ,that describe a data set, and fed into a model which produces a potential outcome. By repeating the process more results are generated until at some point the data can display a pattern that describes the outcome. The result is a quantified probability which describes the chance that something might occur based on the quantity of occurrence generated by the model [31–33].

The Monte Carlo methods can differ depending on the algorithm and application in which it is used, but in summary most implementations will follow a general pattern [32]:

0. Construct a model which is able to describe an outcome of the problem.
1. Define the space of which inputs can be used by the model to get an outcome (creating a parameter space).
2. Use the model to generate results based on random sampled input from the parameter space.
3. Order and determine which results are part of a certain outcome and draw conclusions on the generated statistical evidence.

2.7 The use of the Monte Carlo method and its pitfalls

The Monte Carlo method is widely used within various applications in different fields of science but is suitable for problems; where all inputs are known but it is too inefficient to compute the problem with a deterministic method; that require uncertainty to be incorporated into the analysis or prediction and is suitable for discovering new parameters in a model to improve the current model. Monte Carlo is not good at solving problems where answers are known but the inputs are not and in solving problems where no uncertainty should be in the answer produced.

All mentioned problems it can solve all tend to rely on significant quantities of data which makes it a relative time consuming process for generating results. Meaning of the generated result is highly depended on the model and random sampling technique that is used and both contribute to biases in the results [31, 32, 34].

2.8 Tumor Necrosis Factor Receptor Associated Syndrome

Tumor necrosis factor receptor-associated periodic syndrome (TRAPS) is a rare (1 : 1,000,000) hereditary autosomal dominant disease , formerly known as Familial Hibernian Fever (FHF) [35], that can cause recurring fevers with durations from days up to months. Symptoms during these fevers are: skin rash, swelling, inflammatory reactions across the whole body and pain in the abdomen, muscles and/or joints. A long term and lasting effect is the accumulation of amyloids within the kidneys and may result in other diseases [7]. TRAPS is known to be caused by mutations within the gene tumor necrosis factor receptor 1 (TNFRSF1A/TNRF1) (Section 2.9), of which few mutations are known to hinder the transport of proteins to the cell surface and trap them in cell causing the activation of inflammatory response [7, 36]. So far 158 mutations have been associated with the disease [37], but more mutations have been identified in TNFRSF1A wherein some might be pathogenic (Sections 3.6.1, 3.6.2).

2.9 Tumor Necrosis Factor Receptor Super Family Member 1A

Tumor Necrosis Factor Receptor Super Family Member 1A (TNFRSF1A/TNFR1) is a gene located on chromosome 12 region 1 band 3 and sub-band 31. The gene produces a trans-membrane receptor consisting of 445 residues divided into 221 residue cytoplasmic section and a 171 extracellular part that consists of 4 conserved cysteine rich domains [38–40]. The receptor is ubiquitous across most cell surfaces , but is not found on erythrocytes [41], and can form two different types of unbound hexagonal clusters depending on the dimer formation [42]. When the structures are dimers the binding sites are exposed making it possible for tumor necrosis factor (TNF) α and β (Section 2.10) to bind in homotrimeric form, by binding of TNF the dimers disconnect and three TNFR1s interact with the TNF homotrimer [42]. With the interaction of TNF homotrimers with TNFR1 it can activate several pathways such as; The nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), which enhances the transcription of various genes during inflammation, infection or other forms of external stress; Also it is able to activate the extrinsic pathway of apoptosis by releasing the silencer of death domain (SODD) proteins, on the cytoplasmic site of the cell, which will activate the formation of Tumor Necrosis Factor Receptor type 1-Associated DEATH Domain protein (TRADD) [43] that forms a complex that attracts Fas associated death domain (FADD) [44]. On binding of FADD initiator caspase 8 starts a cascade wherein caspase 3 is activated and will cleave aspartate out of proteins and thereby disrupting the metabolism[45–47].

2.10 Tumor Necrosis Factor Alpha and Beta

The proteins TNF α and β are both pro-inflammatory cytokines that are produced as response to an infection or when a cell is damaged. Both are transcribed from their genes that reside in chromosome 6 in the p-arm at region 2 band 1 and sub-band 3. TNF α and β are 35% identical and 50% homologous to each other consisting out of 233 and 205 amino acid residues. Both are able to form a homotrimeric structures that can bind to the dimeric form TNFR1 (Section 2.9) to activate the extrinsic pathway[39, 48–50].

3 Materials and methods

3.1 VIPUR approach

Variant interpretation using Rosetta (VIPUR) is a machine learning approach for predicting deleteriousness of proteins (loss of function) and uses sequential and structural information. To train it the VIPUR training set (VTS) was made which contained sequential and structural features from protein structures that were acquired from Modbase [51] and SWISS-MODEL [52–56]. Proteins that did not have a structure in modbase or SWISS-MODEL were modeled with Modeller (Section 3.5.1) from proteins that had the highest amino acid sequence identity to the desired protein. based on protein fragments that had the highest amino acid sequence identity to the protein Some structures from the databases had: duplicate chains, ligands, metals and non-standard amino acids which were removed to avoid inconsistencies for the generated features by tools. For all proteins a mutation file was made that described which and how many residues had to be mutated, with this file DDG monomer (Section 3.3.2) could determine changes in the protein structure, most results by ddg were used as features. Structural mutations of proteins that are in the VTS were introduced by a script using PyMOL (Section 3.5.2) by default or PyRosetta (Section 3.5.3) when PyMOL was not available. After a mutation the structure was optimized by the relax application (Section 3.3.1) that produced 50 structures of a single variant, for each of these structures property scores where available of which all quartiles have been used as a machine learning feature. of which the quartiles are used as a learning feature. Probe (Section 3.2.2) calculated the solvent accessible surface area (SASA/ACCP) of a protein in square Ångstrom (\AA^2) which was used as a structural machine learning feature. The sequence features of VIPUR were produced by PSI-blast (Section 3.2.1) on non mutated sequences and blasted against the NCBI protein database (nr) which resulted in a position specific scoring matrix (PSSM). From the PSSM scores of the information content, non-mutated, mutated, the difference in scores between non-mutated and mutated and the difference between groups [2, 57] used as sequential feature for VIPUR. With 106 features generated by the mentioned tools deleteriousness of a protein variant is determined with sparse logistic regression. The term sparse implies that a limited set of features was used because the weights "shrink" to 0 with regularization [58].

3.2 VIPUR specific tools

3.2.1 PSI-BLAST

Position specific iterative basic local alignment search tool (PSI-BLAST) focuses on distant relatives of proteins by making a profile of the sequence and querying it at a protein sequence database. With the generated results a new profile is constructed which is queried to the same database as the previous query. These steps are repeated several times to determine which residues are found in distant relatives and results is a position specific scoring matrix (PSSM) that describes the frequency of which residues are substituted by a specific other residue [59–61]. From the PSSMs sequences features were acquired for the VIPUR machine learning method.

Position-Specific Iterated BLAST 2.7.1+

3.2.2 Probe

Probe is able to evaluate atom packing for a single protein or for interacting proteins. It does this by creating a probe, which is described as a sphere like object, that marks an area with dots when at least two non-covalent atoms are in contact with the probe. [62, 63]. VIPUR used this tool to calculate solvent accessible surface area (SASA or ACCP).

version 2.16.130520

3.3 Rosetta

Rosetta is a software suite that has a variety of tools that are developed to aid in macro molecular and antibody, analysis, design and prediction [64]. However no tools in the suite have been encountered

that could introduce missense mutations in the proteins and was done by other software (Sections 3.5.2, 3.5.3, 3.5.1). With the introduced mutations water had to be removed because some tools cannot predict structures well with: water, metals and amino acids that are no part of the standard (20) amino acids [65].

Within the tools from Rosetta various scores are assigned to different properties related to bonds, interactions, energies and geometries within structures and are written into a score file. From all different scoring metrics the Rosetta score, Rosetta energy unit (REU) or total_score in the score files, can be used to compare models from the same protein with the same tool. Not only is the score based on energy but also it has statistical terms which influence the score based on known favorable folds from existing structures that reside in the curated Rosetta database [23]. In summary to asses models, a lower Rosetta score makes a more natural model.

Rosetta software suite Version 3.10

3.3.1 Relax

The Relax application was used by VIPUR and by SPVAA to relax the side chains to minimize energy levels within the local conformational search space [66] of the structure. It determines the energy levels with a Monte Carlo method (Section 2.7) and after a certain set of moves it produces a structure and starts anew [67, 68].

3.3.2 DDG Monomer

DDG monomer is meant to predict energetic stability of a point mutation in monomeric protein. The application was used by VIPUR to collect features related to energies bonds, bridges and constraints differences between the wild type and a mutated protein. To execute the tool a script had to be ran which renumbered the wild type pdb file and it required a "mutation file" that described the changes of a residues based on name and position[69].

3.3.3 Rescore

With this tool Rosetta scores can be calculated based on PDB files proteins structures [70] , the output is identical to that is written within the score files produced by Relax (Section 3.3.1).

3.3.4 Backrub

The backrub application is based on the Monte Carlo method (Section 2.7), and alters a protein by moving its backbone residues with a strategy called fix end move (FEM). With this strategy, groups of residues are selected at random from the structure that can contain up to: four dihedral, two bond angles and two end points. Both ends of a group are fixated at their position in which a new angle α arises, within this angle residues are pivoted in their natural occurring maximum range of $\pm 10^\circ$ [71, 72]. With this application backbones of newly introduced mutations were altered, for each attempt a new file was generated and the scores were written to a score file. The lowest Rosetta scoring model was selected to undergo side chain relaxation with the Relax application (Section 3.3.1).

3.4 Structure prediction web services

3.4.1 Robetta prediction server

The web tool Robetta integrates several tools to form protein structures with homology modeling (Section 2.4). Its only requirement is an amino acid sequence, but optionally constrains and fragments can be added to disallow movement of certain structures or add known fragments to avoid calculating pieces that are already known. With this information Robetta searches with the help of sequence aligners for known fragments and tries to incorporate them into a single protein structure [73–77]. The known fragments of TNFRSF1A (Section 2.4) were given as a template to Robetta and modeled into a whole

protein to make it possible to introduce mutations and predict pathogenicity of variants.

<http://new.rosettaweb.org/>

3.4.2 I-TASSER prediction server

The I-TASSER web server is a tool that is able to predict protein structures with a FASTA sequence. The first step it takes is finding structural templates which resemble the sequence by local meta-threading server (LOMETS). LOMETS starts with multiple sequence alignment of which several sequences will undergo protein threading by different programs to form structural templates. The templates are assessed made from: the highest alignment Z-score, a program within LOMETS specific confidence score and sequence identity [78, 79]. The known fragments of TNFRSF1A (Section 2.4) were given as a template to I-TASSER and modeled into a whole protein to make it possible to introduce mutations and predict pathogenicity of variants.

Server version, <https://zhanglab.ccmb.med.umich.edu/I-TASSER/>

3.4.3 HOPE

Have yOur Protein Explained (HOPE) is a web service that collects information of about a user specified missense mutation in a protein and comes from various sources. Uniprot (Section 3.7.2) is queried with BLAST to find homologous sequences and structures, other features that are found on Uniprot are active sites, domains and various other sequence features that help to identify the function of a region. From the BLAST results homology models are made with Yasara that are sent off to WHAT IF web services that calculate structural information about the protein. Before the formation of a report all information is put into a decision tree to assess mutational effects in context of: contacts, structural locations, non-structural features, previous variant information and amino acid properties. [3, 80–82]. With this method it is not possible to assess ligands and complexes at once but only a single missense mutation within a protein.
Version 1.1.1, <https://www3.cmbi.umcn.nl/hope/>

3.5 Structural modification and visualization software

3.5.1 Modeller

Modeller is software that is developed for homology modeling but it was used for its utilities which allowed to; complete protein data bank (PDB) structures with missing atoms; predict disulfide bonds that were missing and mutate protein residues [83–86].

Version 9.21

3.5.2 PyMOL

Visualization of 3D structures; making images of proteins; putting monomers in the correct position; replacing TNF β structure with a TNF α in the bound structure and aligning the structures to measure the distance between models and X-ray structures were done with PyMOL [87]. PyMOL was in VIPUR used in combination with Python (Section 3.8.1) to perform mutagenesis on the protein structures.

Version 2.2.3

3.5.3 PyRosetta

Is an application programming (API) which has Python bindings (Section 3.8.1) for the Rosetta software suite (Section 3.3) and finds its use in VIPUR when no PyMOL (Section 3.5.2) was available to mutate residues in a structure [88].

Version 4

3.6 Amino acid sequence variant tables

3.6.1 GAVIN Machine Learning Data Table

Is a collection of nucleotide mutations from rare diseases used by the GAVIN [1] machine learning approach. From this set the genes of TNFRSF1A (Section 2.10) with a missense mutation were filtered (Section 3.8.2) and written into a format which the variant effect predictor could (VEP) [89] could read and translate from nucleotide to protein mutations. The classification of the variants was done by experts based on the five tier IARC classification system [90].

3.6.2 gnomAD

The gnomAD database consists of unified data from large scale genome sequencing data projects and is based on genome reference consortium human genome build 37 human genome 19 (GRCh37/hg19). From this database missense mutations were collected for TNFRSF1A (Section 2.9), no classification was known from these mutations [91].

3.6.3 Infevers

Infevers is a website about auto hereditary inflammatory diseases for which each are tables that contain information about mutations and their classification. The table for TRAPS (Section 2.9) was used to collect missense mutations of TNFRSF1A gene [92].

3.7 Protein functional and structural databases

3.7.1 Research Collaboratory for Structural Bioinformatics

Research Collaboratory for Structural Bioinformatics (RCSB) is a database where whole or fragmented experimentally determined proteins structures, that are published, can be found and downloaded. The Fragments for modeling (Sections 3.4.1, 3.4.2) whole TNFRSF1A (Section 2.9) (1EXT [93]) and determining the differences in energy levels (Section 3.3.1) with TNF α - β (1TNR [40]) with the interaction site were acquired from this database [94].

3.7.2 Uniprot

Knowledge from various omic domains about proteins have been linked together into single database called Uniprot which makes all information accessible at once. For TNFRSF1A (Section 2.10) the FASTA sequences were collected from Uniprot and for structures it redirected to (Section 3.7.1) [95].

3.8 Scripting languages

3.8.1 Python

Both VIPUR and the single protein variant analysis approach (SPVAA) were written in Python. SPVAA was written in Python because of its ease of use and the modeller bindings (Section 3.5.1) that were available. The mutations that were put together from the different tables (Sections 3.6.2, 3.6.3, 3.6.1) with R (Section 3.8.2) were filtered by a Python script. From the mutations a compact list was made by a different script that described the chains that had to be altered by modeller to introduce the appropriate mutation into a PDB file. From these PDB files several were selected to be optimized by pipeline that used backrub (Section 3.3.4) and Relax (Section 3.3.1) to optimize the structure.

Laptop version 2.7.15

Server version 2.7.11

3.8.2 R scripting language

With R the tables from gnomAD, GAVIN and Infevers (Sections 3.6.2, 3.6.1, 3.6.3) of TNFRSF1A missense mutations (Section 2.9) were merged together in a new comma seperated values file with their known classifications. Ordering and filtering the double mutations and removing double classifications where done with Python (Section 3.8.1). It has also been used in combination ggplot2 [96] and data.table [97] to make density plots of all scores acquired from Rosetta Backrub and Relax (See the supplementary for R package versions).

R scripting front-end version 3.5.2 (2018-12-20)

3.9 Utility software

3.9.1 Bash

Unix like operating systems (OS) have a shell which allows users to interact with programs on a computer or with the computer itself based on commands submitted. The default shell for MacOS and also for several Linux distributions is the Bourne again shell (Bash) which was used to launch Python scripts (Section 3.8.1) and submit jobs to the SLURM workload manager (Section 3.9.2).

Laptop Version GNU bash, version 3.2.57(1)-release (x86_64-apple-darwin18)

Server Version GNU bash, version 4.1.2(2)-release (x86_64-redhat-linux-gnu)

3.9.2 SLURM

For computational jobs where a laptop or desktop does not suffice because due to the lack computational resources a computer cluster could come to aid. These clusters consist out of several computers that execute resource intensive tasks, to manage these systems as optimal and fair as possible a workload manager like simple Linux utility resource management (SLURM), is installed. Jobs are submitted that request resources for execution and are scheduled on the systems queue.

3.9.3 MPI

Some tools from the Rosetta software suite (Sections 3.3) have the ability to use multiple central processing unit (CPU) cores from a single computer or from multiple computers. With a message parsing interface (MPI) it is possible for software to communicate between CPU cores on the same and on different computers to exchange information about processes giving the ability to share work between computers and CPUs.

OpenMPI/1.8.8-GNU-4.9.3-2.25

4 Results

Determining the effects of protein variants is not a trivial task and therefore VIPUR, SPVAA and HOPE have described and employed within this order.

The SPVAA scripts can be found at: <https://github.com/Sylt-CSI/variant-protein-prediction>
The VIPUR scripts with applications and data can be found at: <https://osf.io/bd2h4/>

4.1 Reviving the VIPUR approach to expand rare disease diagnostics

4.1.1 Preparatory steps for using the VIPUR approach

After the publication of VIPUR the tools, data and applications became available at the open science framework (OSF) [98] which were downloaded and studied. All applications from the Rosetta software suite available through OSF (Section 3.3) were pre-compiled without support for MPI (Section 3.9.3) and with that could not benefit from multiple CPUs. To solve this a new version of the Rosetta software suite was built with MPI support in a slurm job where the compilation could benefit from multiple CPU cores.

4.1.2 Resolving VIPUR system incompatibilities

Within the VIPUR pipeline residues were mutated to determine the effects of a structural mutation, by default missense mutations were introduced with PyMOL (Section 3.5.2), an alternative from VIPUR was Pyrosetta (Section 3.5.3) when PyMOL was not available. Neither of these programs could be built or compiled because the lack of open graphics library (OpenGL) for PyMOL and having the incorrect C++ and C libraries for PyRosetta. To bypass both programs modeller was introduced which is also capable of replacing residues(Secton 3.5.1).

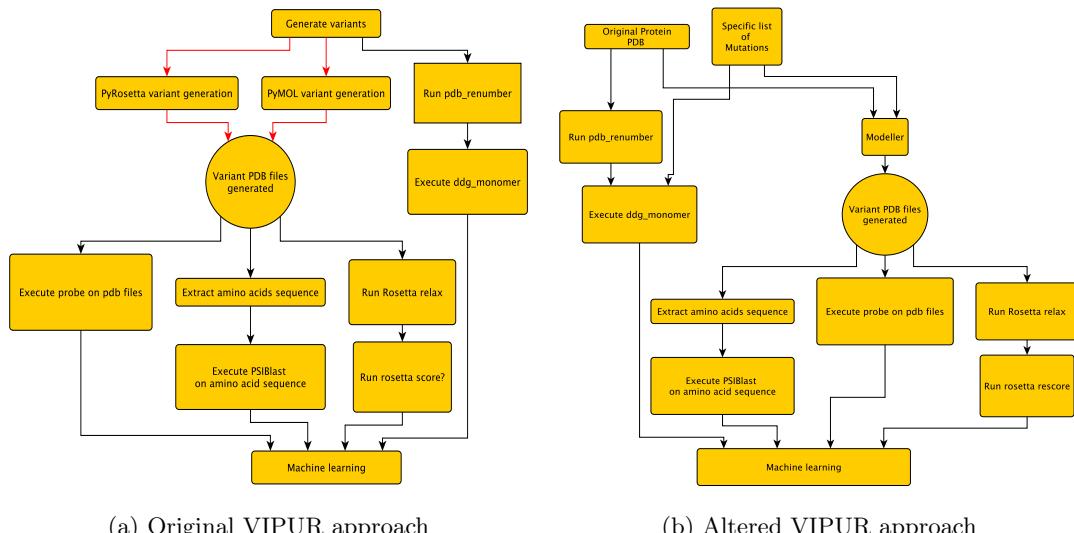


Figure 1: Both flowcharts illustrate the VIPUR pipeline wherein each block is a procedure and each arrows is the path to the up following step. The central circle was the problem that had to be fixed by a different program than PyMOL or API than PyRosetta. Figure 1a has red arrows that indicate that both methods were incapable to produce the mutated PDB files. Within figure 1b the alternative method is proposed wherein PyMOL and PyRosetta (Sections 3.5.2, 3.5.3) are substituted by Modeller (Section 3.5.1) to acquire the mutated protein structures. (To zoom in on the details within the figure it is recommend to look at the PDF version.)

4.1.3 Expanding the VIPUR training set with data from TNFRSF1A by homology modeling and protein threading

Since the VTS did not have any features of TNFRSF1A (Section 2.9) the amino acid sequence was collected from Uniprot (Section 3.7.2) and the structure from RCSB (Section 3.7.1). The structures available of TNFRSF1A were incomplete, fragments for the binding site [40] were available and its death domain that interacts with TRADD [99] (Section 2.9). To acquire a monomeric structure of TNFRSF1A two ab initio modeling web services I-TASSER and Robetta (Sections 3.4.2, 3.4.1) had been employed. Both were given the task to model the whole protein with and without a binding site template to determine how well they could model the structure. Determination of the best model was based on the lowest root mean square deviation distance (RMSD)in Å between produces models and X-ray crystallographic structures of the TNFRSF1A binding site.

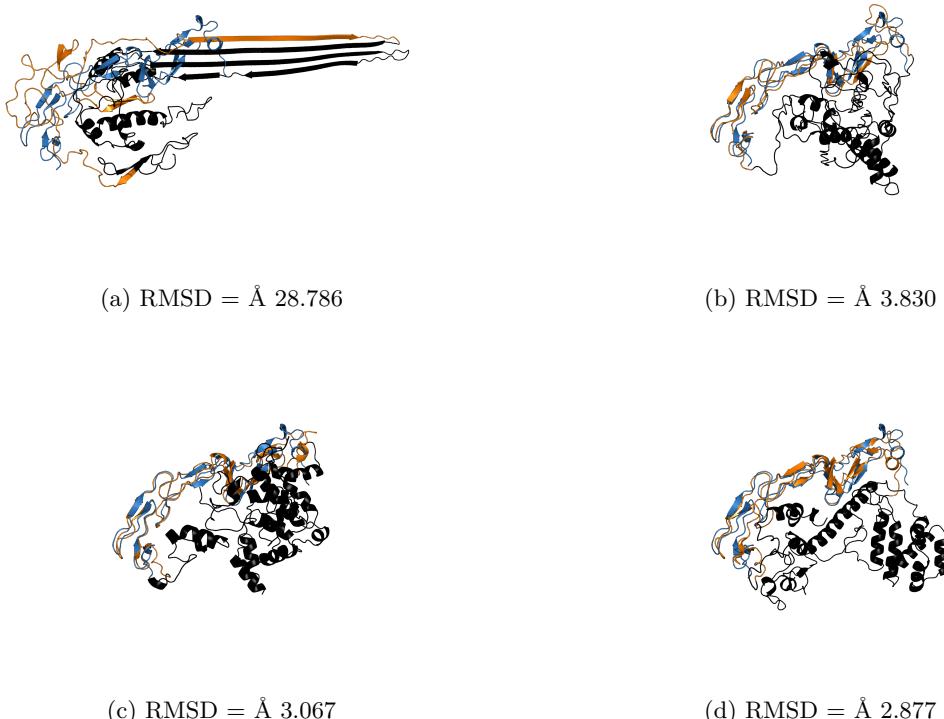


Figure 2: 3D structures of TNFRSF1A (2a, 2b: I-TASSER, 2c, 2d: Robetta) without (left: 2a, 2c) and with templates (right: 2b, 2d). The sky blue colored structure in each figure is an X-ray crystallographic model (1EXT) of the binding site of TNFRSF1A and the orange structure is the representation of that identical fragment in the model made by the web services. (To zoom in on the details within the figure it is recommend to look at the PDF version.)

4.1.4 Practical VIPUR usage

With the hindrance of software incompatibility on the cluster, difference in produced models between the web services, discovery of consequences by removing elements from structures, and the time it would take to reverse engineer VIPUR a new decision was formed. VIPUR would be set aside for now and if time was left it would be modified so that it could evaluate structures.

4.2 Analyses of proteins variants TNFRSF1A

4.2.1 Requirements for determining structural and binding effects of protein variants

Protein variants can be assessed from multiple perspectives and together they can form a holistic view on how a protein works and how mutations affect its workings. However adding perspectives to the protein assessment makes it complex and requires expertise to determine its validity and contribution, therefore the analysis has been limited to basic structural information.

Various proteins consist of multiple chains that can be identical or different depending on their function [100] and should be taken into account when assessing protein variants since one residue might alter the binding between chains and might alter the proteins formation. Different molecules and atoms that do not make up a protein but play a role in a pathway and function (ligands and co-receptors) are able to affect a proteins shape [101] and can behave differently when a residue is mutated.

A different aspect that can change with mutations is alteration in motions between structures which allow or disallow certain movements to occur and inhibit or promote binding.

4.2.2 Introduction of the simple protein variant analysis approach

A different method for analyzing proteins variants than VIPUR is by determining the changes in energy levels (Section 3.3) between a wild type and a variant. To make a variant of the wild type, a structure was required wherein a missense mutation could be introduced with Modeller (Section 3.5.1). The backbone structure of the variant was modified with the backrub application (Section 3.3.4) to make it better interact with other amino acid backbones in the structure resulting in 1000 models. The lowest Rosetta scoring (Section 3.3) structure would be selected to further improve the side chains with the Relax application (Section 3.3.1). With the application 64 models where made of which all scores were plotted in a density plot with R (Section 3.8.2) and the lowest scoring model was visualized with PyMOL (Section 3.5.2). This method is called the simple protein variant analysis approach and was only test on TNFRSF1A (Section 2.9) and its ligands TNF α and β . It shows similarities to VIPUR in the sense that it has to remove waters and metals, but it can keep its ligands and duplicate chains.

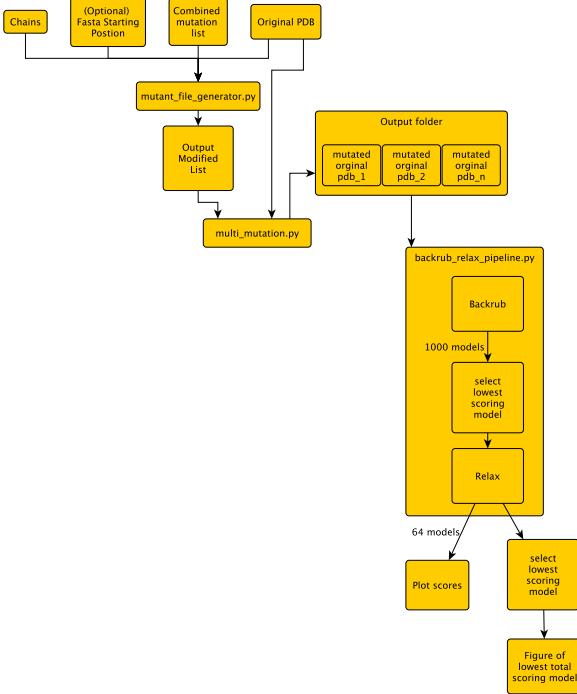


Figure 3: Flowchart of SPVAA wherein a list of known mutations generate the appropriate information for modeller to mutate residues in the original PDB. The mutated models are fed into the backrub relax pipeline to transform the structures into a low energy state. (To zoom in on the details within the figure it is recommended to look at the PDF version.)

4.2.3 Carrying out SPVAA on TNFRSF1A

Before introducing mutations into a protein structure it is helpful to know if a mutation has been observed to avoid allocating resources to mutations that do not occur. Therefore three tables with observed TNFRSF1A mutations (Sections 3.6.1, 3.6.2, 3.6.3) have been combined with an R script (Section 3.8.2) into a single table consisting of two columns. The first column (split into three columns table 1) contains strings that describes the: original residue, position and new residue, the second column describes whether a formed mutation is pathogenic or benign.

Original residue	Position in the protein sequence	New residue	Classification
Cys	44	Tyr	PATHOGENIC
Thr	44	Pro	PATHOGENIC
Thr	44	Ser	PATHOGENIC

Table 1: The format wherein mutations were filtered from the GAVIN, gnomAD and Infevers tables (Sections 3.6.1, 3.6.2, 3.6.3), describe whether a structural mutation is harmful or not, for many mutations in the table the effect is unknown. (To view the whole table visit the supplementary.)

For assessing variants in TNFRSF1A a structural fragment was used that contained TNF β (1TNR) [40] which was made homotrimeric with PyMOL (Section 3.5.2). It resulted in six chains that emulate a bound TNFRSF1A with TNF β . The first column of the mutation table did not contain sufficient information to apply mutations correctly and within the PDB different numbering is used than in the amino acid sequence. To bundle the information and make it usable for introducing mutations a Python script (Section 3.8.1) has been written that combines the mutation table, PDB chains and the correct

position within the sequence into a type of table which has sufficient information to mutate structures.
The script that reads table 1 and produces 2 in combination with a PDB, respective chains and an optional starting position is the mutant_file_generator.py.

Iteration number	Filename	Chain	Residue index in chain	New residue
34	1tnr3_TNFA	R	0	TYR
34	1tnr3_TNFA	T	0	TYR
34	1tnr3_TNFA	S	0	TYR
35	1tnr3_TNFA	R	0	PRO
35	1tnr3_TNFA	T	0	PRO
35	1tnr3_TNFA	S	0	PRO
36	1tnr3_TNFA	R	0	SER
36	1tnr3_TNFA	T	0	SER
36	1tnr3_TNFA	S	0	SER

Table 2: The format that describes the mutations that should be made by Modeller (Section 3.5.1), with specifications of the: model, file, chain, residue index and the new residue. The whole table for TNFA and TNFB are visible within the supplementary.

To introduce mutations within PDB structures a Python script (Section 3.8.1) was written which used the generated mutation table (Table: 2) and a matching PDB structure, from the table. The iteration number specifies if a mutation has to be stored in a single file or across multiple files. The filename serves as key that determines the PDB that should be used. The letters specify chains, numbers are indices within a chain (Python list) and the last column states the three letter code of the new residue. When a structure is read in through the Python bindings of Modeller (Section 3.5.1) all non standard atoms and molecules are removed because Rosetta (Section 3.3) is not able to deal with those atoms. Just before mutagenesis takes place missing atoms are added to the structure that were difficult to determine with experimental methods(Section 2.3). After the introduction of a last attempt was made by modeller to add disulfide bridges on places where a potential connection could be made based on distances between cysteine residues.

The script that is able to produce mutations into PDBs and uses table 2 is multi_mutation.py

With many protein variants generated and limited resources available a few mutations had to be picked to undergo the backrub relax pipeline. All mutations that were analyzed were picked from the Infevers table because these variants are from a single isoform and had a protein structure (1TNR [40]) available that interacted with TNF β . Mutations cysteine 62 to glycine and phenylalanine acid 141 to isoleucine were validated as pathogenic mutations within Infevers table and were used to determine the effectiveness of SPVAA. Within the infevers table no benign validated missense mutations were available [102], but to still have the opportunity to asses a likely benign mutation, the mutation glutamic acid 138 to alanine had been chosen to be assessed with SPVAA.

In the attempt to make mutated structures behave more natural two tools from the Rosetta software suite (Section 3.3) had been used to minimize energies within protein structures. With the Backrub application (Section 3.3.4) 1000 altered backbone models have been produced each with 10000 Monte Carlo moves (Sections 2.7). For each model that Backrub generated a set of scores were assigned to the properties, which together formed a collective score that described energy and bond occurrence in nature (Section 3.3). Models of the wildtypes and mutants with the lowest collective score ,the total score, were chosen to undergo further side chain optimization within the Relax (Section 3.3.1). 64 different relaxed models were produced and with various scores related to the properties of which the one with lowest total score would be chosen to visualize.

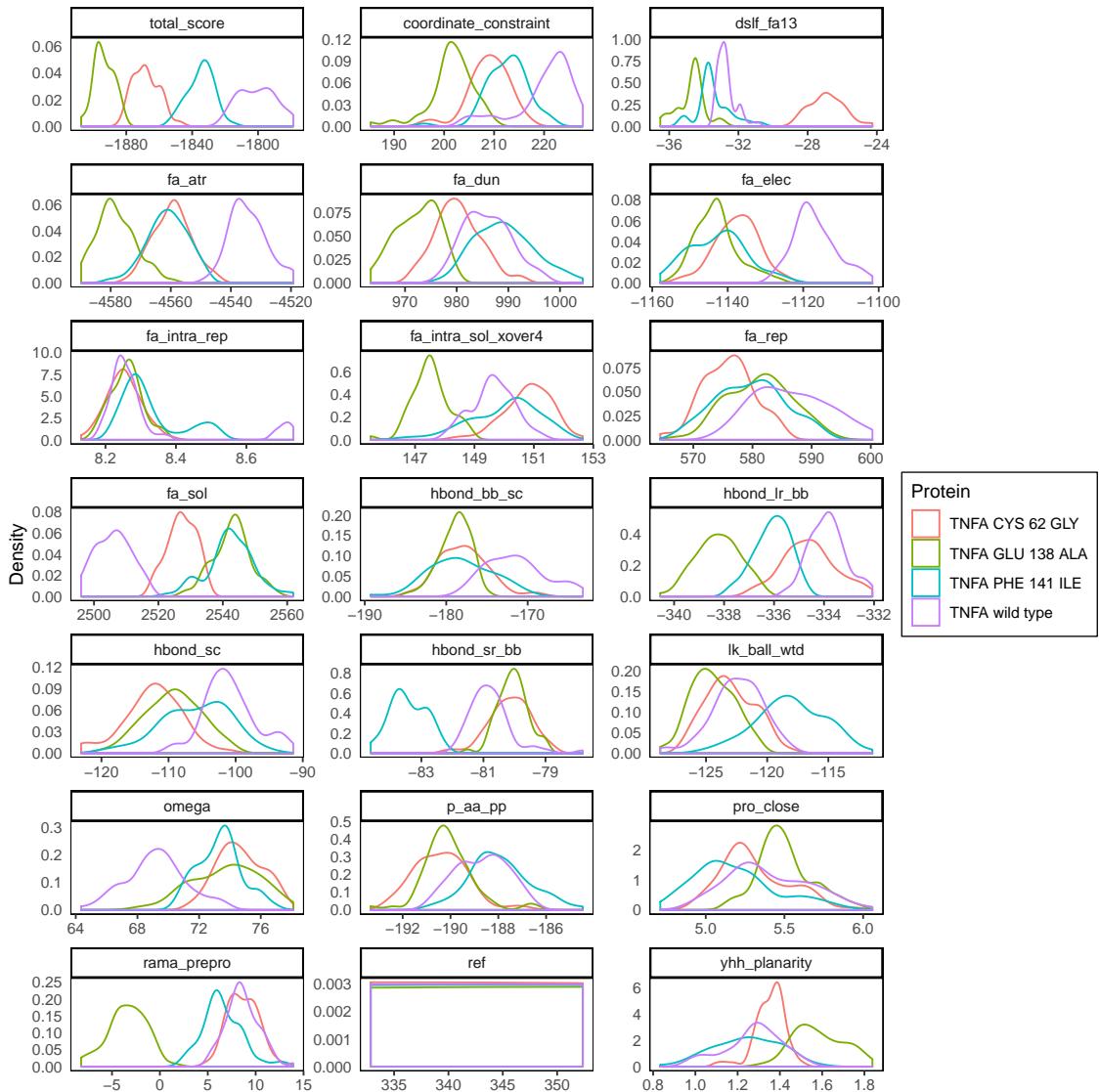


Figure 4: Density of different scoring metrics from the models produced with relax of the wild type and all mutants that interacted with TNF α . Most of the total scores, fa_atr values and fa_ele values of the mutated model are lower than the wild type that is bound to TNF α . The fa_sol values are higher of the wild type than the mutants. TNFA CYS 62 GLY has more higher values at the dslf_fa13 (disulfide geometry potential) than all other mutations. (Plots of backrub TNF α scores are in the supplementary.) (To zoom in on the details within the figure it is recommend to look at the PDF version.)

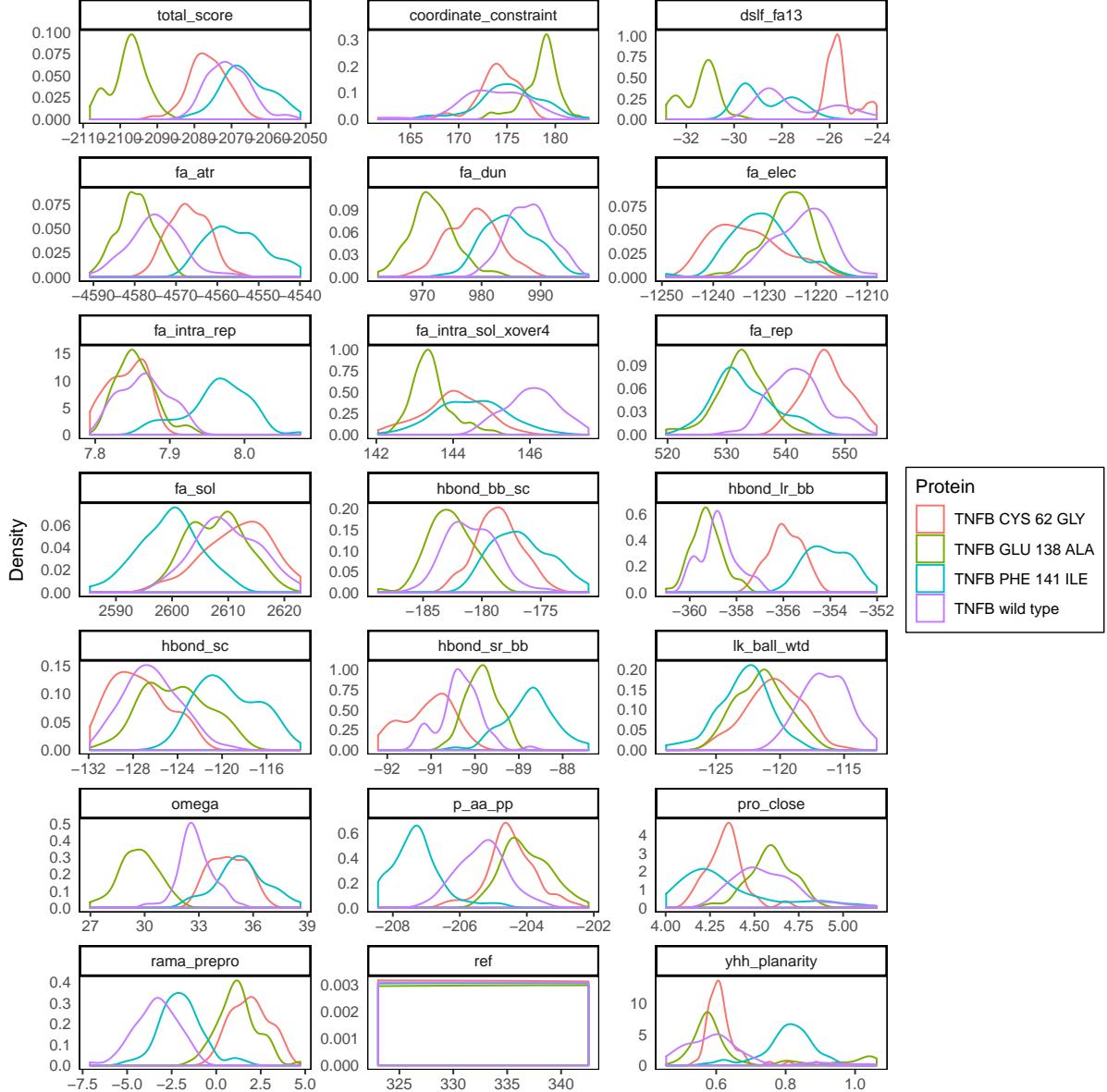


Figure 5: Density distributions of the scores generated by relax for the wild type and mutations of TNFRSF1A that interact with $\text{TNF}\beta$. The total score values of TNFB GLU 138 ALA are lower than all other models and dslf_fa13 (disulfide geometry potential) values are higher at TNFB CYS 62 GLY than the other models. fa_intra_rep (Lennard-Jones repulsive between atoms in the same residue) is higher within the models of PHE 141 ILE and hbond_lr_bb (Backbone-backbone hbonds distant in primary sequence) is higher at CYS 62 GLY and PHE 141 ILE. (Plots of backrub $\text{TNF}\beta$ scores are in the supplementary.) (To zoom in on the details within the figure it is recommend to look at the PDF version.)

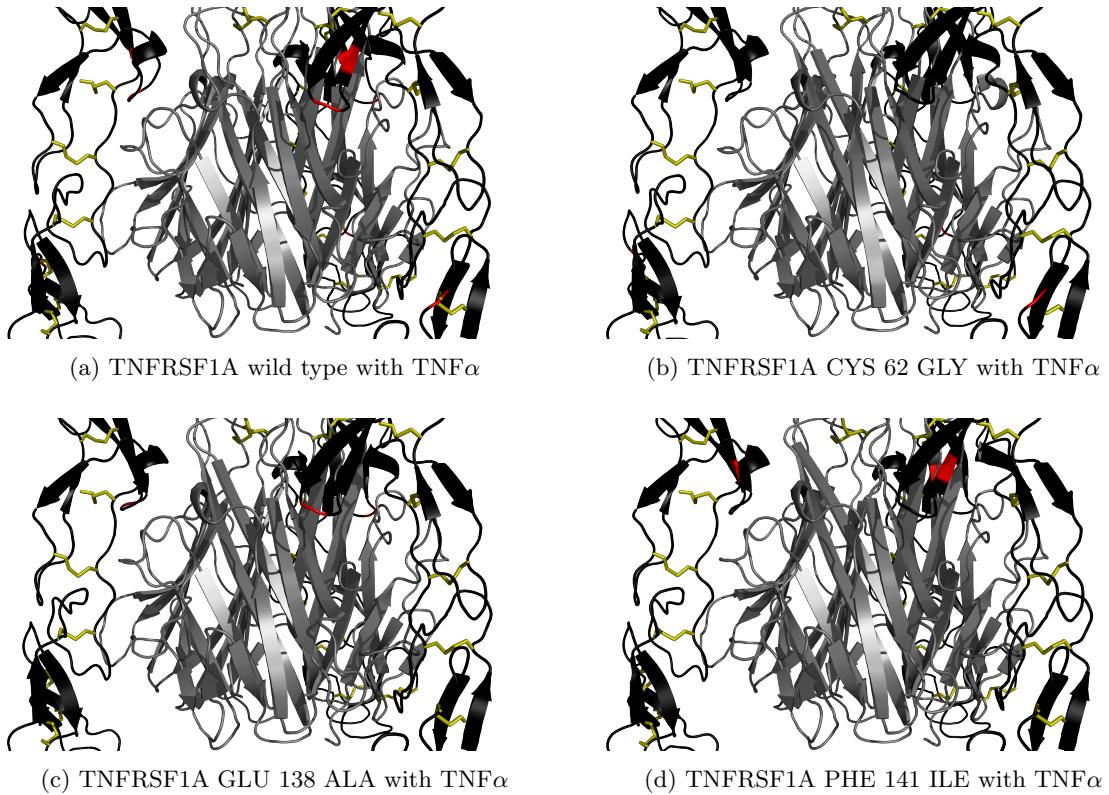


Figure 6: 3D structures of a homotrimer TNFRSF1As (black) with a homotrimer TNF α s (gray) and disulfide bridges (dark yellow). The wild type (6a) has three red colored areas which are the original residues of the protein before any form of mutation. Within CYS 62 GLY (6b) it is visible that at the position where a mutation is introduced (red) a disulfide bridge is missing. The mutations of GLU 138 ALA (6c) and PHE 141 ILE (6d) show no large differences at the mutated spots (red). (To zoom in on the details within the figure it is recommend to look at the PDF version.)

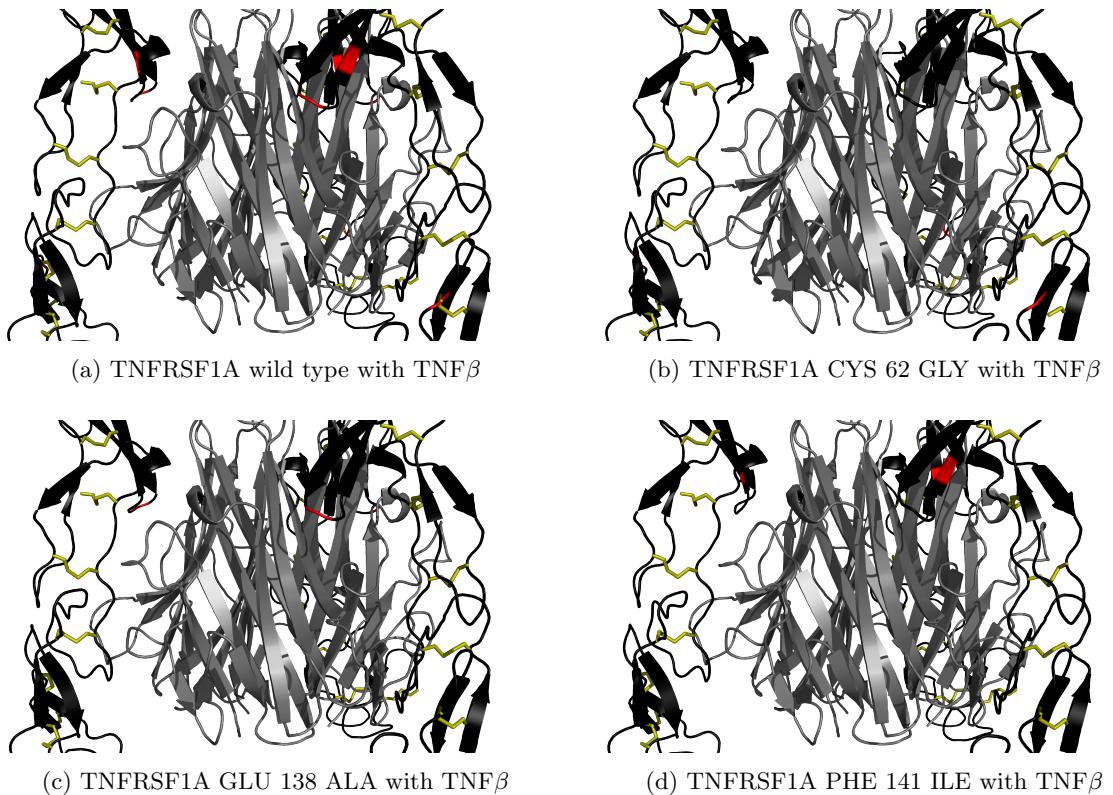


Figure 7: 3D structures of a homotrimer TNFRSF1As (black) with a homotrimer TNF β s (gray) and disulfide bridges (dark yellow). The wild type (7a) has three red colored areas which are the original residues of the protein before any form of mutation. Within CYS 62 GLY (7b) it is visible that at the position where a mutation is introduced (red) a disulfide bridge is missing. The mutations of GLU 138 ALA (7c) and PHE 141 ILE (7d) show no large differences at the mutated spots (red). (To zoom in on the details within the figure it is recommend to look at the PDF version.)

4.3 Finding mutation information with HOPE

A more textual informative approach is web service HOPE (Section 3.4.3) which makes a report about the mutation. The mutations that were known from Infevers (Section 3.6.3) and were also used with SPVAA were tested by HOPE (CYS62GLY, GLU138ALA and PHE141IIE). The reports are visible within the supplementary.

HOPEs first test was the mutation of Cysteine 62 to glycine, which is known within the Infevers table as pathogenic and was validated. It discovered that the residue was involved in a disulfide bridge and was 100% conserved in related protein sequences, based on the observation that cysteine formed a disulfide bridge it expected that with the replacement of it glycine would make the whole structures less rigid. HOPE predicted that mutation is pathogenic because of the high conservation of the residue, which is further confirmed by its search results in which it found the original publication of the discovery that it has been associated to TRAPS [37].

According to Infevers is the mutation of glutamic acid at position 138 mutated to alanine classified as likely benign and was not validated yet. HOPE discovered with a BLAST query that glutamic acid occurs often at position but other residues such as alanine have been observed at the position. Structurally glutamic acid forms salt bridges with proline 368 and leucine 390 and is found in a sequence of amino acids that is repeated through out TNFRSF1A. The amino acid lies within a domain where it interacts with other domains and is important for the proteins activity, with this mutation it might already perturb the binding capabilities according to HOPE.

The last mutation that was tested with HOPE was phenylalanine 141 to Isoleucine and was according to Infevers pathogenic and has been validated. Phenylalanine is conserved at this positions and few other residues have been seen at the position, it is a member of the identical domain as glutamic acid 138. HOPEs prediction was not clear if this mutation could cause any harm.

5 Discussion

Currently, most rare disease patients do not receive a molecular diagnosis. Despite machine learning methods such as GAVIN that can remove 95% of benign variation from the genome[1], it is still very difficult to pin-point causal variants in the genome. Such methods rely mostly on evolutionary conservation and have been heavily optimized over the years. Therefore we need new refreshing approaches such as VIPUR, which uses sequential and structural data instead, that have the exciting potential to help us diagnose more patients.

5.1 VIPUR

Within the attempt to make VIPUR usable for diagnostics it was discovered that some questionable steps were taken to make it applicable for diagnosis but also to determine deleteriousness ; (i) "All protein models were standardized to remove unwanted components (duplicate chains, ligands, metals and non-standard amino acids)" [2]. Standardizing data can be beneficial to avoid learning features from proteins that are available to some models but should not be the determining factor for classification. However any form of context to the protein is removed and might therefore make incorrect assumptions about how: a monomer interacts with other monomers, ligands, metals, non-standard amino acids and water which can all have an effect on how proteins shape and interact [101]. (ii) With the utilization of Rosetta's Relax application different models are formed based on the Monte Carlo method (Section 2.7). VIPUR produces 50 structures with Relax per protein which is a tiny amount of the potential search space of possible folds that could have made changes in a mutated protein, which is also visible in the scores of the models made from of TNFRSF1A with TNF α & β (Figures 4, 5). Rosetta itself suggests to make sufficient models, starting with a minimum of 5000[103]. (iii) The features acquired with probe in combination with the models that were produced, within the publication of Probe is mentioned: "It requires both highly accurate structures and also the explicit inclusion of all hydrogen atoms and their van der Waals interactions." [62]. It is not possible to determine if the structures were accurate. However is it likely that no loose hydrogen atoms were included within the structures because all were standardized. To make the outcome of Probe useful to VIPUR the program Reduce should have been executed first, which adds hydrogen atoms to the structure. It is recommended on the site of Probe to execute Reduce on the structure before using VIPUR [63].

More questions arise when further investigating the publication. Within the figures (4, 5) [2] and supplementary figures (10, 11) [58] are heatmaps of PSSMs added that display the values of the natural and mutated residues. In combination with the methods used on standardizing structures and collecting features a suspicion arises that there is little contribution from the structural features and that prediction depends on PSI-BLAST results. Figure 4 within the publication shows a protein wherein serine 204 which part of an α helix is mutated to proline and is predicted as deleterioueness by VIPUR. Which is logical even without predictions because prolines are known to be α helix stoppers [104] and therefor affect the form of an α helix (which is also not visible in the figure.).

VIPUR has not been used for various reasons. The models that were predicted by the web services (Figures 2) had a decent accuracy for the binding site, but the rest of structures differed and made it too difficult to determine which model was an accurate representation of TNFRSF1A. VIPUR was built on and for a single system and required reverse engineering to make it work in any form even without the substitution of PyMOL and PyRosetta (Sections 3.5.2, 3.5.3) with Modeller (Section 3.5.1). The differences in models is probably due to the fact that TNFRSF1A is transmembrane protein which is hard to acquire structures from with experimental methods [24], even though $\sim 57\%$ of TNFRSF1As structure was known (1EXT[93] , 1ICH[99]).

Although it is not part of developing a technique that can help diagnosing rare disease variants but the publication contains a claim ("VIPUR can be applied to mutations in any organism's proteome...." [2]) which contradicts with its methods: "remove unwanted components (duplicate chains, ligands, metals and non-standard amino acids)" [2]. Currently there are more than 140 amino acids found in natural proteins of which 22 are part of the amino acid alphabet and 20 of those are classified as standard [105].

By removing the non-standard amino acids from proteins it becomes impossible to analyze mutations in every organisms proteome.

5.2 SPVAA

SPVAA did not assess whole complex and neither did became a machine learning tool ready to use in diagnosis to make automated predictions. SPVAA has similar weaknesses as VIPUR wherein: water, metals and other molecules are removed from its structure. However the proteins can keep their extra monomers and protein ligands, even when the structure requires identical or different ones they can be added manually into the structure and analyzed.

For three variants it has been attempted to acquire structural information to determine their differences with the wild type structure, in two of the three structures hardly any changes were visible. From the models that were assessed only homotrimeric TNF α - β bound mutations were processed, not the unboud dimeric structures. From the proteins that were modeled too few were produced of each structure. Backrub used 10000 Monte Carlo moves, which is very little compared for the amount of residues it has and should have had more to make larger changes in the structure. The relax application made 64 models per mutation but Rosetta itself suggest to make at least 5000[103]. The only models with mutations where pathogenicity was highly likely visible were the CYS 62 GLY models, that had broken disulfide bridges that could lead to instability in the protein. Based on the scores produced by Relax little could be discovered except the higher scores in the disulfide geometry potential (dslf_fa13) of CYS 62 GLY. Many of the scores show overlap and are difficult to relate with the applied structural changes. It could have been that many of the relax score distributions should have had more overlap with each because too few models were made.

The models could have had in other situations better disulfide bridge formation which did not rely on the guess of distance between cysteine residues. A better method which Modeller has is to form disulfide bridges based on other protein data that is available in some situations. Within TNFRSF1A it likely did not matter too much because except CYS 62 GLY all other methods show identical disulfide bridges.

5.3 HOPE

HOPE is an informative tool that is easy to use, fast and makes structural problems within proteins understandable when a missense mutation is discovered. However it does not draws a solid conclusions and the information it collects depends on: previous publications, conservation and experimental structures. HOPE has a disadvantage when limited knowledge is available. Also it does not asses a complex but it can describe binding sites from the monomer when previously discovered. For the mutation CYS 62 GLY it was very clear based on conservation and the publication that it was pathogenic. However for the pathogenic mutation PHE 141 ILE the information was less clear. Glutamic acid at position 138 makes salt bridges according to HOPE which are in general strong bonds and can be important for internal structures and binding. With this information GLU 138 ALA would be likely pathogenic, however it does not draw a conclusion. Also no change in structure or interaction has been observed with HOPE or SPVAA and therefore can not be classified pathogenic with certainty and would therefor remain likely benign.

6 Conclusion

All used methods use structural information acquired from experimentally determine structures which are mostly fragments due to the difficulty and expenses of determining structures. Because they are mostly fragments and miss context it can be hard to make relevant assumptions about some protein structures or its general effect in a cell.

At its current state VIPUR is not usable for diagnosing defects within proteins and most likely also not for predicting deleteriousness. To make it usable for predicting effects of variants or deleteriousness it could benefit of manual curation of all wild types, which is partially fulfilled by using proteins from the SWISS-MODEL database. A different addition would be looking at the structure in context of its environment since that is where the wild type should behave properly. With that information proper predictions can be made about the deleteriousness of a protein or its pathogenicity.

SPVAA analyses proteins more into a natural context than VIPUR because it allows the use of more chains and ligands. However the assessment of structures made by SPVAA require expertise to determine the effects of mutations and at its current state it is not user friendly or helpful to inexperienced users. CYS 62 GLY was the only mutation that without prior knowledge could be assumed pathogenic based on the plots and models (Figures 4, 5, 6b, 7b) that were produced. None of the other mutations contained clear information whether they would be pathogenic or not and hardly any differences have been observed between TNF α - β .

HOPE is an informative tool that gave new insight in the mutation GLU 138 ALA, in some cases it can be very clear and almost form a conclusion but in other situations it is unable to discover effects of a mutation to elucidates its user and makes the dependence of previously investigated knowledge visible.

7 Future work

The VIPUR approach could be investigated to test whether the features generated by PSI-BLAST are the main predictors. To measure PSI-BLAST feature importance within VIPUR it first needs to be reverse engineered to make it work with Modeller or another tool which is able to implement mutations in PDB files. Once the reverse engineering is finished VIPURs feature importance can be tested with shap [106–112] that explains the output of machine learning models and or with similar methods and software on the VTS.

SPVAA is currently highly dependent on the resources that are available and comes short to produce enough models [103]. One option to improve the quantity of models is by using different software that is less resource intensive. Another option would be to run SPVAA on a different cluster that has more nodes and allows to setup jobs that use multiple nodes. SPVAA is not a prediction method and has to be modified and expanded become a variant predictor that is able to predict pathogenicity or deleteriouness. A good starting point for such a predictor would be according to the guidelines in section 2.5.

VIPUR and SPVAA could both be improved in various ways, one of them would be by doing molecular dynamic simulations on the mutated structures to determine the effects of structural changes. With SPVAA it would have most likely become clear if the loss of the disulfide bridge from CYS 62 GLY in TNFRSF1A would have caused structural issues. VIPUR could benefit from molecular dynamics as new machine learning feature in situations where limited movement is observed and it changes tremendously when a missense mutation occurred in a protein increases with a mutation or vice versa.

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Supplementary