# AutoCaSc: Prioritizing candidate genes for neurodevelopmental disorders

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**Abstract**

Routine exome sequencing (ES) in individuals with neurodevelopmental disorders (NDD) remains inconclusive in >50%. Research analysis of unsolved cases can identify novel candidate genes but is time consuming, subjective, and hard to compare between labs. The field therefore needs automated and standardized assessment methods to prioritize candidates for matchmaking. We developed AutoCaSc (https://autocasc.uni-leipzig.de) based on our candidate scoring scheme (CaSc). We validated our approach using synthetic trios and real in-house trio ES data. AutoCaSc consistently (94.5%) scored variants in valid novel NDD genes in the top three ranks. In 93 real trio exomes, AutoCaSc identified most (97.5%) previously manually scored variants while evaluating additional highly scoring variants missed in manual evaluation. It identified candidate variants in previously undescribed NDD candidate genes ( *CNTN2*, *DLGAP1*, *SMURF1*, *NRXN3*, *PRICKLE1*). AutoCaSc enables anybody to quickly screen a variant for its plausibility in NDD. After contributing >40 descriptions of NDD associated genes, we provide usage recommendations based on our extensive experience. Our implementation is capable of pipeline integration and therefore allows screening of large cohorts for candidate genes. AutoCaSc empowers even small labs to a standardized matchmaking collaboration and to contribute to the ongoing identification of novel NDD entities.

# INTRODUCTION

Routine genetic analyses and exome sequencing (ES) are able to clarify the cause of neurodevelopmental disorders (NDD) in about 30-50% of affected individuals(Trujillano et al., 2017; Deciphering Developmental Disorders Study et al., 2020; Shashi et al., 2014; Yavarna et al., 2015; Chong et al., 2015). Due to the extreme heterogeneity of NDD (1,534 primary genes in the SysID(Kochinke et al., 2016) database as of 2021-12-06), this diagnostic yield will increase with identification of novel genetic associations. (Chong et al., 2015; Eilbeck et al., 2017; Kaplanis et al., 2019)

Large screening studies have focused on the identification of *de novo* variants (DNV) in NDD (Vissers et al., 2010). The DDD (Deciphering Developmental Disorders) (Deciphering Developmental Disorders Study et al., 2020) study evaluated 31,058 trio-exomes of individuals with NDD with the aim to identify causal DNVs. By mere statistical enrichment, 28 novel genes (10.0% of 281 total enriched genes) could be associated with NDD. Cases from the DDD cohort were subsequently contributed to several descriptions of novel NDD associations based on clinically and functionally guided matchmaking. Thus it is clear that a more distributed approach is needed that goes beyond analyzing ever large cohorts of only a few centers, one that assesses gene and variant characteristics in, at best, all unsolved trio exomes to identify further NDD entities.

In principle, any rare variant segregating with a disorder can cause a disease. (Najmabadi et al., 2011; Tarpey et al., 2009; Vissers et al., 2010) Wet lab or model organism analyses of all candidate variants would be slow and expensive. Therefore, a systematic procedure for pre-selection of the most promising candidates is needed to close the gap between the large number of candidates and the experimental proof of their causality. Scientific evaluators usually consider aspects of predicted effect on the protein, segregation in the family, minor allele frequency in population databases like gnomAD (Genome Aggregation Database Consortium et al., 2020), functional aspects of the protein and other information from literature and from public sources. (MacArthur et al., 2014) This approach is highly evaluator dependent, making it both subjective and not scalable. Furthermore, it is time-consuming and needs expert knowledge, thus deterring many labs from scientific evaluation of their ES data. A standardized and quick evaluation in a measurable framework is therefore needed.

Computational approaches have been developed to predict novel disease genes based on confirmed gene associations (e.g. ToppGene Suite (Chen et al., 2009), Endeavour (Tranchevent et al., 2016)). Other approaches use evolutionary constraints to predict the importance of a gene for the organism in general (e.g. pLI (Exome Aggregation Consortium et al., 2016)) or the deleteriousness of single genetic variants (e.g. CADD (Kircher et al., 2014)). Few projects (eXtasy (Sifrim et al., 2013), PHIVE (Robinson et al., 2014), Phenolyzer (Yang et al., 2015), OMIM explorer (James et al., 2016), DeepPVP (Boudellioua et al., 2019)) use both variant and gene-specific information for candidate prioritization; a quick review and comparison of the tools combining gene and variant data for candidate gene prediction, showed that they are often unmaintained, unusable or do not offer a visual user interface in the first place (compare Table S1).

Here we describe the development of a candidate scoring scheme (CaSc) specifically intended for the NDD research field and based on commonly used complementary data sources for genes and variants. The webAutoCaSc web tool enables broad applicability while the command line tool vcfAutoCaSc is designed for screening cohorts and pipeline integration. We validated our approach using synthetic trios and real in-house trio ES data. The successful application of these principles and tools at our Institute resulted in >40 descriptions of rare NDD entities. Based on this experience, we recommend a workflow for re-evaluation of sequencing data in unsolved NDD trios.

# MATERIALS AND METHODS

## The Leipzig NDD research cohort

An ongoing study at the Institute of Human Genetics at Leipzig University Medical Center investigates the genetics of rare diseases using high throughput sequencing (also commonly referred to as Next Generation Sequencing or NGS). This study was approved by the ethics committee of the University of Leipzig, Germany (224/16-ek and 402/16-ek) and all individuals or their parents or legal guardians consented to genetic testing.

All individuals received high throughput sequencing. Genomic DNA was enriched using either a clinical exome target design (CES; TruSight One v1 panel, 4811 genes, Illumina, Inc., San Diego, USA) or different exome target designs (TWIST Human Core Exome Kit, TWIST Bioscience, San Francisco, CA, USA; BGI Exome capture 59M kit, BGI, Shenzhen, China; SureSelect Human All Exon V6, Agilent Technologies, Santa Clara, California; TruSeq DNA Exome and Nextera Rapid Capture Exomes, Illumina, Inc., San Diego, CA, USA). Resulting libraries were sequenced with paired end reads (100bp or 150bp) on either Illumina (NextSeq 550 or NovaSeq 6000) or BGI sequencers (BGISEQ-500). Most samples were initially sequenced using an affected only ("single") approach. The data was first analyzed in a diagnostic setting. In unsolved cases with research consent and parental DNA samples, we subsequently complemented the analysis by trio ES and re-evaluated the case.

Research re-evaluation in unsolved cases included specially trained staff at our institute manually re-checking the data to identify candidate genes and variants based on gene annotations and literature/ database review.

Training and supervision were performed by principal investigators with long lasting experience in molecular genetics diagnostics and NDD research.

## Establishing a comparable candidate score (CaSc)

In order to standardize this approach and make results comparable throughout genes and by different analysts, we established a candidate score (CaSc) as a set of criteria to evaluate variants in novel candidate genes for NDD. Roughly, the set of criteria included variant attributes (missense or loss-of-function (LoF) variants, *in silico* (computational) prediction tools, conservation, minor allele frequency), inheritance aspects (segregation and zygosity, and the relation to higher pLI-scores(Exome Aggregation Consortium et al., 2016) for LoF variants and missense Z-scores (Genome Aggregation Database Consortium et al., 2020)), and the gene's plausibility for causing NDD. This was calculated based on reviewing OMIM and PubMed entries to check if the gene is involved in neurological processes, using MGI (Bult et al., 2019) and PubMed for animal models, STRING (Szklarczyk et al., 2019) for protein interactions, GTEx (Consortium, 2020) for gene expression in central nervous system, as well as a list of resources to find out if variants in the gene have been described in association with autism spectrum disorder or NDD (the resources included the DDD study, the Human Gene Mutation Database (HGMD), ClinVar, cooperation partners, and entries in GeneMatcher). An overview of the manual version of CaSc is provided in Figure S1 and a detailed description in the corresponding preprint (Büttner et al., 2019). Evaluators manually applied these rules to all candidate variants and focused on following up the highest scoring and thus most promising genes through matchmaking. Evaluating a variant took between 5 and 15 minutes. As we moved on to the automatic version of CaSc, there have been several changes to the scoring logic (see below and detailed in the Supplementary notes).

## Automatic Candidate score (AutoCaSc)

The manual scoring process showed to be suboptimal regarding time needed and inter-evaluator variability when applied without extensive prior training and constant supervision, which is hard to maintain in a university setting with continuous workforce change. We thus decided to develop a software tool to remove subjectivity and enable lasting comparability and continuous quick re-evaluation of older candidate genes. AutoCaSc is written using the Python programming language and all code is publicly available through a GitHub repository (https://github.com/JohannKaspar/AutoCaSc) under a creative commons license (CC BY-NC-SA 4.0).

The three categories "Inheritance", "Gene constraint" and "Variant attributes" were implemented as simple decision trees based on an updated CaSc logic (detailed in Table S2). For the "Gene plausibility" category, we reviewed public databases providing information on gene expression, annotated literature, animal models and reported variants. Criteria for using a resource were the uniqueness of the used data sources, its completeness and regular updates or sustained maintenance. We selected the Genotype-Tissue Expression (GTEx (Consortium, 2020)) project for expression data, Mouse Genome Informatics (MGI (Bult et al., 2019)) database for model organism data, STRING (Szklarczyk et al., 2019) database for protein-protein interactions, PubTator Central (PTC) (Wei et al., 2019) for annotations of PubMed abstracts, DisGeNET (Piñero et al., 2017) for gene disease association (GDA) and PsyMuKB (Lin et al., 2019) for reported DNVs. DisGeNET is a partially redundant source because it calculates GDA based on published literature and from various repositories and databases. However, we found that the integration of DisGeNET overall improves the gene plausibility score as it includes data beyond literature.

When submitting genomic or transcript coding variants to AutoCaSc, they are annotated using the Ensembl Variant Effect Predictor (VEP) (McLaren et al., 2016) via the Ensembl REST (representational state transfer) API (application programming interface). Latest allele counts are queried through the GraphQL API of gnomAD (Genome Aggregation Database Consortium et al., 2020). Gene constraint metrics such as pLI and Z-score(Exome Aggregation Consortium et al., 2016), predicted impact of the variant on protein function, mode of inheritance, GERP++ (Davydov et al., 2010) rankscore (RS), frequency in gnomAD (Genome

Aggregation Database Consortium et al., 2020), SIFT (Kumar et al., 2009) converted RS, MutationTaster (Schwarz et al., 2014) converted RS and MutationAssessor (Reva et al., 2011) RS are then used to calculate the variant score. This is then summed with the precalculated gene plausibility score. Please refer to Figure 1b, Table S2 and Table S3 for an overview of all criteria and data sources used.

We provide webAutoCaSc (https://autocasc.uni-leipzig.de/) as an online implementation. The webtool (user interface mock in Figure 1d) is intended for quick manual scoring of a few variants. It does not store any query information on the server side. All server access logs are deleted on a daily basis using a cron command.

The command line implementation of our algorithm (vcfAutoCaSc) uses slivar (Pedersen et al., 2021) to pre-filter variants in multi-sample VCF (Danecek et al., 2011) files and automatically scores all passing variants. Family information in the standard PED pedigree format can be provided as input. This allows AutoCaSc to be implemented in existing pipelines for rare variant analysis where it can be used to automatically prioritize candidates in large datasets.

### Synthetic trio benchmark

To evaluate AutoCaSc, we searched for recent publications in high quality genetic journals describing novel NDD associated genes. We curated a list of 79 variants in 11 novel NDD genes described in 9 publications from a total of 21 reviewed publications (File S1). We reasoned that due to their novelty at the time of review (2021-01-18), these would be comparable to candidate variants that a research lab would evaluate. Next, we used two publicly available trios of healthy individuals (CEU and ASH trio from Genome In A Bottle (GIAB) (Zook et al., 2016)) and incorporated the reviewed variants into these VCFs files according to their segregation. We then applied vcfAutoCaSc to these synthetic benchmark trios and evaluated the performance by ranking the passing variants by their CaSc. For the scope of our evaluation, we defined two variants in compound heterozygous state as one candidate finding.

### Reevaluation of trio ES

We used AutoCaSc to re-evaluate 93 recent ES trios (TWIST target design) from our in-house NDD cohort. Evaluation in a diagnostic setting (Zacher et al., 2021) using the browser-based "Varvis" genomics software (Limbus Medical Technologies GmbH, Rostock, Germany) revealed a pathogenic SNV in 16 trios (17.2%) and a pathogenic CNV in three trios (3.2%). In the remaining cases, no diagnostic variants were reported and manual analysis in a research setting including the application of the manual version of CaSc was performed. To evaluate the performance of vcfAutoCaSc, we produced a cohort multi-sample VCF file (see Supplementary notes) and annotated it using the above described pipeline of slivar and vcfAutoCaSc.

## RESULTS

### Candidate yield using CaSc

Between January 2016 and December 2020, we evaluated 2,977 cases of individuals with NDD at the Leipzig Center for Rare Diseases and clarified 1,055 of these (35.4%). In 1,922 (65.6%) individuals no clinically relevant variant was identified. Of these, 1,192 cases (62.0%) were reevaluated in a research setting, mostly as trios (1,038; 87.1%) and using CaSc for variant prioritization.

Overall, in 932 families we identified 1,561 candidate variants in 1,309 genes. From these we contributed to 43 publications describing novel NDD entities and are currently working on the clinical and molecular description of 91 candidate genes. A complete and regularly updated list of our in-house candidates(Abou

Jamra, Rami & Platzer, Konrad, 2022) can be found online. In 569 families (61.1%), we identified one candidate gene, in 218 (23.4%) we identified two candidate genes, in 137 (14.7%) we identified three to five candidate genes, and in 9 families we identified 6 or more candidate genes (most of these were homozygous in consanguineous families).

## Novel NDD genes are highly ranked in synthetic trios

We applied vcfAutoCaSc to 158 (79 CEU and 79 ASH based) synthetic trios, containing novel NDD-causing variants from recent publications (Figure 2a). The number of variants remaining after prefiltering varied between 5 and 26, depending on the base trio (CEU or ASH), the sex of the index individual and the parents' affected status.

CaSc of the inserted variants varied between 4.8 and 10.8 with a median of 8.0, whereas the median of all other rare variants, which passed the prefiltering in the trios was 4.3. Of all inserted variants, 75% had a CaSc equal to 6.7 or higher. Comparing true positive and false positive rates, a CaSc cutoff of 6.0 seems optimal (Figure 2b). In the CEU trio, inserted variants were ranked as the top variant in median (mean rank 1.5; top rank in 47/79 (59.5%); second rank in 26/79 (32.9%); third rank in 4/79 (5.0%)). In the ASH trio, inserted variants were ranked as the second highest variant in median (mean rank 2.3; top rank in 0/79 (0%); second rank in 63/79 (79.7%); third rank in 7/79 (8.9%)). The variant that consistently ranked first in the ASH trio was a *de novo* variant in *DNMT3A* present in the HG002 sample.*DNMT3A* is a known NDD gene and the variant type would be typical for the associated disorders (MIM #618724 or MIM #615879, compare Figure S2 for detailed discussion). In 147/158 (93.0%) of simulations, the inserted variant (or the compound-heterozygous candidate finding) was in the three highest scoring candidates. Comparing the filtered ranks of the inserted vs. all other trio specific variants passing the prefiltering showed that the inserted variants were scored significantly higher (ASH Trio: p = 7.5e-17; CEU Trio: p = 1.4e-05; Wilcoxon rank-sum test). Compare File S1 for complete results of the synthetic trio scoring.

## Identification of novel candidate variants beyond confined manual evaluation

We applied vcfAutoCaSc to 93 trio exomes from our in-house cohort. The male to female ratio in this sub-cohort was 57:36 (1.58). Eight (8.6%) families self-reported consanguinity.

Automated filtering and scoring identified 309 unique candidate variants (median 2.0, average 3.3 per case) in 289 genes. The maximum number of candidates per case was 22 variants (1/93 cases; 1.1%) and the minimum was zero variants (15/93 cases; 16.1%). Most (79/81 variants, 97.5%) manually scored candidates were also scored by vcfAutoCaSc, including all 16 SNVs and indels that had been reported to treating physicians Figure 2c). In addition, 230 further candidates, which had not been manually scored before, passed all pre-filtering steps and were automatically scored (Figure 2c). In 15/93 cases (16.1%), no variant passed the filters. In 42/93 (45.2%) cases, the variant evaluated highest by AutoCaSc was also considered in the manual evaluation. In 35/93 cases (37.6%), it was not evaluated manually. In nearly half (24/35; 68.6%) of these cases, there was no single manually evaluated variant reported, but at least one scored by AutoCaSc. The overall highest candidate score in these 24 cases was 10.0, while the median of the highest score was 5.5 in these cases. The number of candidates in these cases varied between one and 10 (median 2). A table with all evaluated variants can be found in File S2.

## Exemplary cavities in automatic filtering

One of the 79 variants (ENST00000159111:c.288C>T p.(Gly96=), *KDM4B* , CaSc 10.0) in the simulation experiment was filtered out by slivar during prefiltering because it was not predicted to lead to a change in the amino acid sequence of the protein (silent) and the impact on protein function was predicted to be low. This variant has been implied as pathogenic by Duncan and colleagues(Duncan et al., 2020) because RNA

analyses showed a splice donor loss combined with the gain of a new donor site (r.287_317del) which was predicted to cause a frameshift at protein level (p.Glu97Thrfs*66).

One of the 81 previously manually scored variants (ENST00000513312: c.487C>T, p.(Arg163Trp), *MCIDAS* , CaSc 6.2) from the in-house cohort was filtered out, because the corresponding gene*MCIDAS* already has an associated non-NDD phenotype (Ciliary dyskinesia, #618695). Genes for which an associated phenotype is noted in OMIM but which are not listed as NDD (candidate) genes in SysID are scored by vcfAutoCaSc but not included in the ranking (compare filtering steps in the Supplementary Methods). Secondly, a pair of variants in compound heterozygous state ((1) ENST00000250937: c.446C>G, p.(Pro149Arg) and (2) ENST00000250937: c.224T>G, p.(Val75Gly), *DOHH* , CaSc 4.4) did not pass slivar VCF quality filters as the read depth (DP = 17) of variant (1) was below our defined cutoff of 20.

### Example of candidate variants identified through automated scoring

Several known NDD genes, such as *SCN1A* , *PIK3CA* ,*CLTC* , *FOXP1* or *SOX5* , were among the strongest candidate variants (Table 1).

The highest scoring variant in a gene currently with unclear association to NDD (according to SysID and PanelApp as of 2021-11-27) was a homozygous LoF variant in *CNTN2* (ENST00000331830: c.940C>T, p.(Arg314*), CaSc 11.4). The predicted impact of the variant was very high (CADD 34.0) and it did not occur in gnomAD.*CNTN2* is highly expressed in the brain (GTEx), and directly interacts (STRING) with known NDD associated genes like *CNTNAP2*(Strauss et al., 2006) and *L1CAM* (Rosenthal et al., 1992).*CNTN2* encodes contactin-2 which, together with the *CNTNAP2*gene product, is responsible for organizing voltage-gated potassium channels at juxtaparanodal regions. (Stogmann et al., 2013) Seizures are described in knock-out mice (MGI). Stogmann and colleagues (Stogmann et al., 2013) have linked a homozygous frameshifting variant in a consanguineous family to familial adult myoclonic epilepsy; of the five affected siblings in this published family, at least two had borderline intelligence, and two individuals had average neuropsychological test scores. In our case, the male individual had epileptic encephalopathy and developmental delay and his brother, segregating the LoF variant, was similarly affected while a healthy sister did not carry the variant in homozygous state. Taken together, an impairment of neuronal functions through biallelic loss of *CNTN2* seems plausible justifying the high score and this gene as a good NDD candidate. Interestingly however there is one one homozygous *CNTN2* LoF variant (c.1169C>A, p.(Ser390*)) listed in gnomAD. Together with the high consanguinity in the case described here this may point to additional aggravating variants ("dual diagnosis") in this family (e.g. homozygous variants in *SELL* with CaSc of 8.09, *FRAS1*a SysID primary gene and *ERMARD* a SysID candidate gene).

Other high scoring variants with previously undescribed or unclear associations to NDD at the time of data retrieval were *DLGAP1* ,*HDAC4* , *H3F3A* , *ANKRD17* , *SMURF1* , *NRXN3* ,*PRICKLE1* and *CASC5* (compare Table 2 for detailed sub-scores). Members of our institute have contributed to a recent publication associating heterozygous LoF variants in *ANKRD17* with a NDD entity based on a cohort of 34 individuals from 32 families (Chopra et al., 2021, p. 17) and pathogenic variants in the histone 3 family (*H3F3A* and *H3F3B* ) have been in the meantime published to cause a NDD entity with neurodegeneration (Bryant et al., 2020). For a complete list of all candidates found in the real trios, see File S2.

# DISCUSSION

Matchmaking platforms such as GeneMatcher (Sobreira et al., 2015) have transformed international collaborations for identifying novel gene-disease associations (GDA) by pooling the results of many genetics laboratories. Thus, very rare GDA can be confirmed, which would be unlikely in the cohort of a single laboratory.

The question, which candidates should be uploaded to matchmaking platforms remains a challenge, since as

the number of uploaded candidates increases, so does the effort required to track and follow up all matches. In terms of specificity, it also makes sense to pre-select the candidates present per individual analysis. AutoCaSc offers an approach to accelerate and systematize this pre-selection in cases of NDD.

In recent years, consistent application of CaSc for candidate gene prioritization at the Center for Rare Diseases in Leipzig has contributed to the identification of 43 new NDD genes, with an additional 91 candidates in ongoing projects and submissions. Considering the relatively small cohort size of just under 3,000 cases of NDD, this is a high yield of new GDA, exemplifying that focusing resources on the most promising candidates is worthwhile.

To demonstrate utility beyond this anecdotal single center experience, we used synthetic trios showing that the programmatic implementation of AutoCaSc prioritizes pathogenic variants in very novel NDD associations with high confidence. In the vast majority (147/158, 93.0%) of simulations, the inserted pathogenic variant was among the three highest scoring variants. As a prospective, real-world benchmark we compared the results of previous manual expert application of the CaSc criteria with the automatic results from AutoCaSc using in-house trio ES. Again, the AutoCaSc filtering and scoring pipeline performed on par with expert curation and identified nearly all (79/81, 97.5%) manually evaluated variants. Based on our validation experiments and long term user experience, we demonstrated that AutoCaSc has a high sensitivity to identify potentially causative candidate variants in genes not yet associated with NDD and that it is able to score a high number of candidates in a short time. It is unbiased and systematically runs the same procedure for all variants that meet certain quality standards and are eligible by inheritance. When applied with similar pre-filtering criteria or on single pre-selected variants, it largely eliminates subjectivity and enables cross-laboratory comparability.

The CaSc score is designed to rank candidate variants in a single analysis or in cohorts of individuals with NDD and thus does not have a universal cutoff. However, candidate variants with a CaSc of >6 were most promising in our synthetic trio experiments and also in our practical use this cutoff seemed reasonable. In the trio simulation, 85.9% of the inserted variants and 16.3% of the trio specific variants were above the CaSc >6 threshold, which is also supported by the receiver operating characteristic curve (ROC) for this experiment (Figure 2b). A cutoff of 5 would result in higher sensitivity and basically identify all true inserted variants, albeit with a higher false positive rate. A high CaSc >9 typically indicates a very good candidate that likely already has an active GeneMatcher collaboration.

While manual curation is time consuming and limited to only a few variants per case, AutoCaSc automatically scored and ranked a further 230 candidates passing prefiltering in the real trio benchmark. A possible reason why these variants were not manually considered for scoring by the human evaluators, is that these did not at first sight seem promising enough to score to the time-limited evaluators. This hypothesis is in agreement with the fact that the majority of these variants received a relatively low score by AutoCaSc. Another possibility is that the evaluators identified publications on the candidate gene that seemed to exclude it as a causal factor; this could be, for example, refuted associations or associations with different disorders but without a NDD phenotype. For example, in the *TrioReal_66* case, a candidate was scored by vcfAutoCaSc that was not documented manually. This was a *de novo* missense variant in *HDAC4* (ENST00000345617: c.1792G>C, p.(Glu598Gln), CaSc 10.0). *HDAC4* is listed in SysID as a known NDD gene. Wheeler and colleagues(Wheeler et al., 2014) demonstrated that haploinsufficiency of *HDAC4* does not cause mental retardation. Based on this, the variant might not have appeared convincing to the evaluators leading to it not being scored. However, certain missense variants in *HDAC4* were recently described to cause a syndromic NDD entity and a gain-of-function effect was discussed based on nucleocytoplasmic mislocation of the protein (Wakeling et al., 2021, p. 4). The variant in *TrioReal_66* affects a different protein region, which is however highly conserved and represents a structured alpha helix in the AlphaFold protein model of HDAC4. Together with multiple *in silico* tools predicting a detrimental effect, its *de novo* occurrence and the high constraint for missense variation of HDAC4 this variant could now be classified as likely pathogenic. This example shows that human evaluation can incorporate more complex concepts like refuted associations, which are currently not implemented in AutoCaSc. It also shows that manual evaluation introduces unreproducible

bias, which can lose interesting variants for follow-up. Reproducible automatic scoring of all filtered variants instead enables research labs to keep an eye on future publications. As it can repeatedly update candidate gene scores at basically no additional cost, AutoCaSc can also be used for continuous re-evaluations of cases to incorporate new knowledge and recent NDD literature which is impossible to do manually. This will be possible with future regular updates and versioning to the score.

While we show the superiority of automated candidate scoring through AutoCaSc, our current implementation has some cavities. AutoCaSc has been validated for trios and lacks functionality for affected only sequencing or more complex family structures like duo or quad approaches. It is possible to score variants with unknown inheritance and segregation, but these variants artificially score low. Also, AutoCaSc missed one of the reviewed *KDM4B* variants in the simulated trios because the pre-filtering removed the variant which was annotated as silent change. This exemplifies that the scoring, especially in vcfAutoCaSc, works only as well as the upstream software and databases. If annotation software incorrectly classifies a variant as irrelevant, it will not be adequately analyzed. Interestingly, this same variant also evades scoring by a recently published decision tool for the PVS1 ACMG criterion (Xiang et al., 2020). Further two variants previously scored manually in the real trios were missed. One was filtered out after scoring by vcfAutoCaSc because the corresponding gene was already associated with a phenotype which was not linked to NDD. We implemented this known disease blacklist filter to remove the high scoring impact of well known (e.g. many publications and associations in the literature) and thus highly investigated genes on filtering results, as well as to remove known reappearing local artifacts (e.g. mucin genes). The second variant was removed in pre-filtering by slivar because its read depth was below our defined cutoff of 20x read coverage. By relaxing the quality settings for prefiltering, more variants could be scored by AutoCaSc if a higher expenditure of time for scoring is accepted. The speed is currently limited by the APIs of VEP and gnomAD, which AutoCaSc uses to retrieve data. By using these, AutoCaSc requires very few resources on the server side and is always up-to-date. If the goal is to apply AutoCaSc to thousands of trios, it should be considered to install VEP and gnomAD locally to avoid the bottleneck introduced through rate limiting of these APIs.

Faster scoring would also allow pre-filtering to be less strict, more variants to be scored, and quality filters to be manually adjusted in the results table, leaving a reasonably large set of candidates. Future implementations and updates to our tools could integrate fast annotation tools like slivar not only for pre-filtering but directly to provide information needed in the scoring process instead of relying on APIs. Future versions of the AutoCaSc tools will allow for sequencing designs beyond trio exomes (single, duo, quad). Furthermore, cosegregation can currently be entered as a supporting argument in the command line version only but will be implemented in the webtool with the next update. Its modularity makes AutoCaSc flexible to easily integrate*in silico* tools with better performance or other omics resources in the future. The web interface also offers possibilities for expansion and automation. For example, a submission to GeneMatcher or ClinVar and sharing of scoring results from authenticated sources would be possible if requested and adopted by the user community.

In summary, we suggest that AutoCaSc should be integrated into existing ES filtering workflows (as depicted in Figure 1a) and the gene scores should be used to prioritize for follow-up. The various interfaces of the AutoCaSc tools will facilitate this integration. Assessing the NDD association of a candidate variant in our framework does not require in-depth literature and database review nor programming knowledge. AutoCaSc can be implemented, in principle, in the routine of all genetic labs doing NDD genetic diagnostics with minimal additional cost. With widespread continuous usage and subsequent upload of the most promising candidate genes to matchmaking platforms like GeneMatcher, we strongly believe it can accelerate the identification of novel monogenic causes of NDD.

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## AUTHORS' CONTRIBUTIONS

B.P., B.B. and R.A.J conceived the initial study concept. B.B. and R.A.J. developed the manual CaSc concept. J.L. programmed the AutoCaSc tools and performed the computational analyses under the supervision of B.P. and R.A.J.. B.B. and R.A.J. created the pre-print draft (https://doi.org/10.1101/588517), concept and figures. B.P. performed bioinformatic analyses to align, call and annotate the exome files used in the validation experiments. J.L. and B.P. created Figures 1, 2 and Table 1, 2 and the Supplementary materials. C.K. and K.P. performed the manual CaSc annotations and aided in the development of the score. B.P, J.L. and R.A.J wrote and edited the manuscript. All authors reviewed and commented on the final draft manuscript.

## AVAILABILITY OF DATA AND MATERIALS

All data relevant to this manuscript can be found in the online version of this article at the publisher's website or has been uploaded to Zenodo (File S1, File S1: https://doi.org/10.5281/zenodo.6190031).

## SUPPLEMENTARY FILES

Supplementary notes: supplementary methods, results, figures and tables, references and file links.

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# TABLES

## Table 1 | Variants in know NDD causing genes found in the 93 trio cohort

## Table 2 | Variants in genes not yet associated with NDD

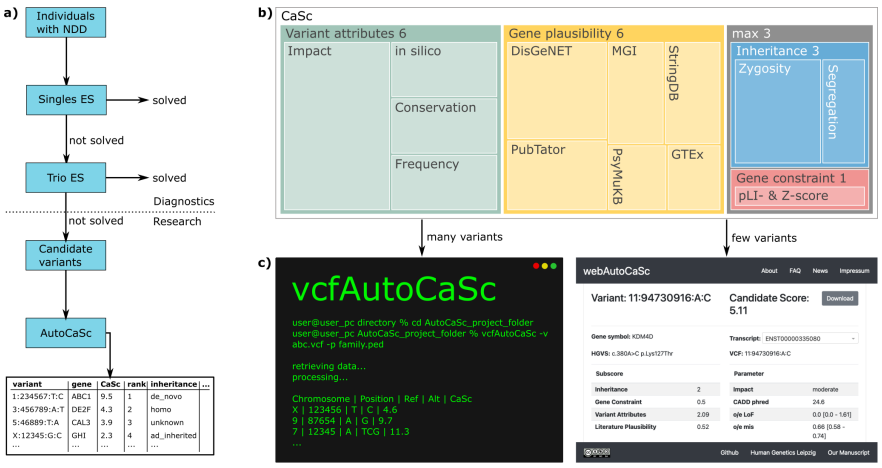
# FIGURES

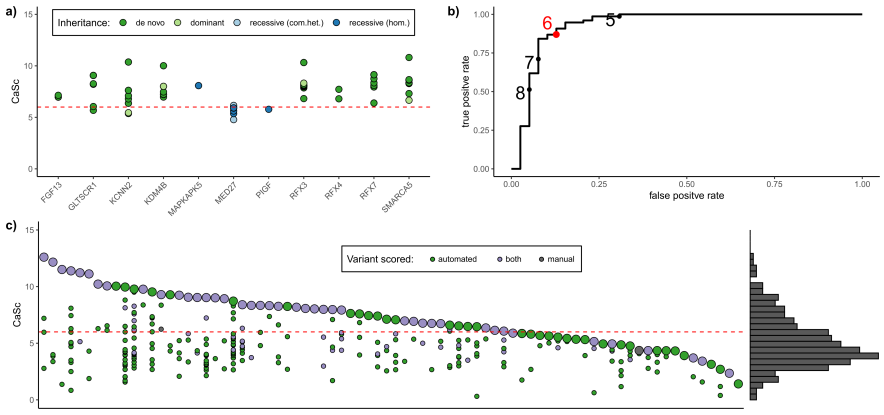
## Figure 1 | AutoCaSc workflow and categories

**a)** Schematic of the proposed workflow embedding AutoCaSc into a genetic pipeline for identification of pathogenic variants in individuals with NDD. When trio exome sequencing does not lead to a diagnosis, AutoCaSc is intended to prioritize variants and thus focus on the most promising candidates. **b)** Composition of the CaSc with the four categories, as well as the maximum achievable points: Variant attributes (max. 6 points), Gene plausibility (max. 6 points), Inheritance (max. 3 points), Gene constraint (max. 1 point). The maximum number of points for inheritance is awarded for autosomal recessive variants where the variant occurs in at least one other affected sibling. Gene constraint metrics, however, are only awarded for autosomal dominant inherited variants, which is why both categories together score a maximum of 3 points. **c)** Depiction of the AutoCaSc tools front-ends. Schematic illustration of vcfAutoCaSc, which is intended for when whole VCFs or cohorts are to be screened (left side). Screenshot of the results page of webAutoCaSc, which is intended to evaluate single or small groups of variants quickly and without installation (right side).

## Figure 2 | Simulated and real trio scoring

**a)** Achieved CaSc of the inserted variants, subdivided according to the affected novel NDD gene. The color of the circles encodes the mode of inheritance. The red dashed line represents a possible cutoff of 6. **b)** ROC curve relating the false positive rate and true positive rate for the simulation experiment. optimal cutoff at CaSc 6 marked in red. **c)** Scoring results for the 78/93 (83.9%) in-house trio exomes with at >0 candidate variants scored. In the left part of the figure, the trios are plotted on the x-axis in descending order of the highest candidate score (CaSc), the y-axis shows the CaSc. The dots represent candidate variants. The large dots are the variants with the highest score in a trio. The colors indicate whether a candidate was scored only manually (n=2), only by AutoCaSc (n=230), or by both (n=79). The right part shows a histogram of the achieved CaSc.



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