Neurotherapeutics
https://doi.org/10.1007/s13311-021-01064-z

ORIGINAL ARTICLE

Drug Repurposing: A Network-based Approach to Amyotrophic Lateral Sclerosis

Giulia Fiscon[1,2] · Federica Conte[1] · Susanna Amadio[3] · Cinzia Volonté[1,3] · Paola Paci[1,4]

Accepted: 2 April 2021

Abstract

The continuous adherence to the conventional "one target, one drug" paradigm has failed so far to provide effective therapeutic solutions for heterogeneous and multifactorial diseases as amyotrophic lateral sclerosis (ALS), a rare progressive and chronic, debilitating neurological disease for which no cure is available. The present study is aimed at finding innovative solutions and paradigms for therapy in ALS pathogenesis, by exploiting new insights from Network Medicine and drug repurposing strategies. To identify new drug-ALS disease associations, we exploited SAveRUNNER, a recently developed network-based algorithm for drug repurposing, which quantifies the proximity of disease-associated genes to drug targets in the human interactome. We prioritized 403 SAveRUNNER-predicted drugs according to decreasing values of network similarity with ALS. Among catecholamine, dopamine, serotonin, histamine, and GABA receptor modulators, as well as angiotensin-converting enzymes, cyclooxygenase isozymes, and serotonin transporter inhibitors, we found some interesting no customary ALS drugs, including amoxapine, clomipramine, mianserin, and modafinil. Furthermore, we strengthened the SAveRUNNER predictions by a gene set enrichment analysis that confirmed modafinil as a drug with the highest score among the 121 identified drugs with a score >0. Our results contribute to gathering further proofs of innovative solutions for therapy in ALS pathogenesis.

Key Words: Drug repositioning · Network Medicine · Amyotrophic lateral sclerosis · Modafinil · Histaminergic drugs

Highlights

- ALS is a rare upper and lower motor neuron disease with no cure insight.
- New ALS-associated repurposable drugs modafinil, amoxapine, clomipramine, mianserin are identified, prioritized by SAveRUNNER network-based algorithm.
- The repurposable drugs predicted by SAveRUNNER are further investigated by gene set enrichment analysis (GSEA).
- SAveRUNNER/GSEA algorithm introduces an integrative framework for the identification and future clinical assessment of ALS drugs.

✉ Cinzia Volonté
cinzia.volonte@cnr.it

Giulia Fiscon
giulia.fiscon@iasi.cnr.it

Federica Conte
federica.conte@iasi.cnr.it

Susanna Amadio
s.amadio@hsantalucia.it

Paola Paci
paci@diag.uniroma1.it

Introduction

Many notions have been disclosed and more evidence is currently claimed about amyotrophic lateral sclerosis (ALS), a rare progressive and chronic debilitating motor neuron disease that impairs voluntary muscle control and movement, and that gathers the attention of scientists and clinicians since the past two hundred years [1, 2]. The main pathological hallmarks of the disease are as follows: damage to upper and lower motor neurons spanning from the motor cortex,

[1] Institute for Systems Analysis and Computer Science "A. Ruberti", National Research Council (IASI–CNR), Via Dei Taurini 19, 00185 Rome, Italy

[2] Fondazione per la Medicina Personalizzata, Via Goffredo Mameli, Genova, Italy

[3] IRCCS Santa Lucia Foundation, Preclinical Neuroscience, Via Del Fosso di Fiorano 65, 00143 Rome, Italy

[4] Department of Computer, Control, and Management Engineering Antonio Ruberti (DIAG), Sapienza University, Rome, Italy

Published online: 13 May 2021

brainstem, and spinal cord [3–5]; defects in neuromuscular junctions causing disassembling and denervation of skeletal muscle [6]; and gradual muscle atrophy leading to strength reduction and alteration of the contractile apparatus [7]. All these processes are accompanied by inflammation caused by toxic factors secreted by activated T cells, gliosis sustained by activated macrophages/microglia/astrocytes, and demyelination triggered by damaged Schwann cells and oligodendrocytes [8].

There are no doubts that all these features make ALS a hot topic and a challenge for investigation. However, the heterogeneous and multifactorial nature of ALS that is characterized by a complex interplay among multiple genetic and environmental factors, together with its low incidence in the worldwide population, can discourage further research, particularly by those pharmaceutical companies mostly interested in capitalizing on human diseases. In addition, the conventional "one target, one drug" paradigm has not provided effective therapeutic solutions for ALS that remains an undefeated disease in the search of new markers and a cure [9, 10].

In the pursuit of alternative and more efficacious treatments, a promising strategy relies on drug repurposing for the identification of novel uses (outside the scope of original medical indications) of drugs already approved by the US Food and Drug Administration (FDA) [11, 12].

To identify drug repurposing opportunities, very promising insights come from the newly emerging field of Network Medicine, which applies concepts and tools from network theory to elucidate the relationship between structural properties of the human interactome (i.e., the integrated network of all physical interactions within a cell), its functional organization, and consequences of its perturbation [13–18]. In the Network Medicine construct, diseases are rarely caused by a single gene mutation, but more typically by the deregulation of a network of genes interconnected to each other. In this innovative vision of human diseases, the interactome can be interpreted as a map, and diseases as local perturbations. In this map, genes that are associated with the same disease tend to aggregate within specific network neighborhoods, or "disease modules" [14, 19]. Similar to the effects of a disease, also the actions of drugs can be interpreted as local perturbations of the interactome and, as a consequence, drug targets that are closer to a specific disease module tend to be more effective for that disease [13, 20–22].

In the present study, we adopt a new network-medicine-based algorithm for drug repurposing called SAveRUNNER (Searching off-lAbel dRUg aNd NEtwoRk) [23, 24] for predicting potential off-label use of drugs in ALS. By quantifying the interplay between ALS-associated genes and drug targets in the human interactome, we identify new drug-ALS disease associations.

Methods

Data Retrieval

The human protein–protein interactome was downloaded from Cheng and coauthors [21], where the authors assembled their in-house systematic human protein–protein interactome with 15 commonly used databases with several types of experimental evidence (e.g., binary PPIs from three-dimensional protein structures; literature-curated PPIs identified by affinity purification followed by mass spectrometry, Y2H, and/or literature-derived low-throughput experiments such as BioGRID [25], HPRD [26], MINT [27], IntAct [28], InnateDB [29]; signaling networks from literature-derived low-throughput experiments; kinase-substrate interactions from literature-derived low-throughput and high-throughput experiments). This version of the interactome is composed of 217,160 protein–protein interactions connecting 15,970 unique proteins.

Disease-associated genes were downloaded from Phenopedia [30], which collects gene associations for 3255 diseases (released 27-04-2020).

Drug-target interactions were acquired from DrugBank [31], which contains 13,563 drug entries including 2627 approved small molecule drugs, 1373 approved biologics, 131 nutraceuticals, and over 6370 experimental drugs (released 22-04-2020). The targets Uniprot IDs provided by DrugBank were mapped to Entrez gene IDs by using BioMart – Ensembl tool (https://www.ensembl.org/). For some drugs of interest for which no targets were found in DrugBank, we integrated drug-target interactions available from the Therapeutic Target Database [32].

Study Design

The pipeline of our analysis can be summarized as follows (Fig. 1). Given as input, (i) the human interactome network, where nodes are molecular components of human cells and a link occurs if a physical interaction exists among them, and (ii) the lists of disease-associated genes, we applied the Random Walk with Restart (RWR) algorithm to build the human disease network, where nodes are diseases and a link occurs between ALS and other diseases if, starting from the ALS disease module, the other disease module is more likely to be reached by a random walker on the interactome (see "Random Walk with Restart Algorithm"). Next, given the same input data of the RWR algorithm jointly with additional drug-target interactions, we applied SAveRUNNER algorithm. SAveRUNNER searched for drugs that could be a repurposable candidate for ALS disease by exploiting the vicinity between the drug modules and the ALS disease module in the human interactome network (see

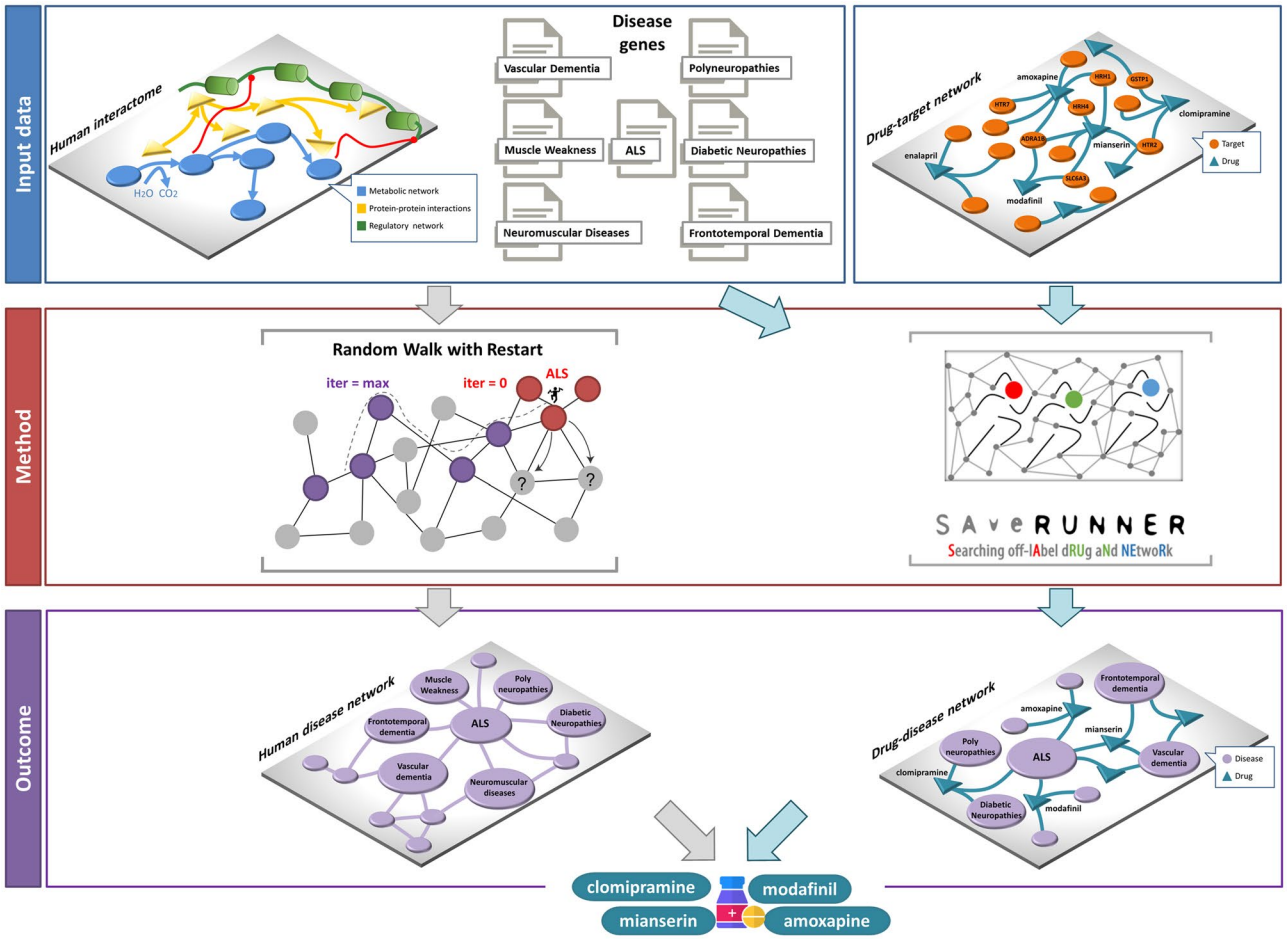

Fig. 1 Pipeline of the analysis. The analysis proceeds by following two branches. [Left, grey arrows] The human interactome network obtained from [21] and the lists of disease genes retrieved from Phenopedia [30] for the analyzed disorders (blue box, Input data) were given as input of the Random Walk with Restart (RWR) algorithm (red box, Method), which provided as output (purple box, Outcome) the *human disease network*, where nodes are diseases and a link occurs between two diseases if, starting from one disease module, the other one is more likely to be reached by a random walker on the interactome. [Right, turquoise arrows] The human interactome network, the lists of disease genes, and the drug-target interactions downloaded from DrugBank [31] (blue box, Input data) were given as input to SAveRUNNER algorithm (red box, Method), which provided as output (purple box, Outcome) the *drug-disease network*, where nodes are FDA-approved drugs and diseases, and a link occurs if a drug is predicted to be repositioned for that disease. Among the drugs predicted by SAveRUNNER as potentially repurposable for ALS disease, we focused on those drugs that are histaminergic modulators and whose original medical indications referred to diseases connected to ALS in the human disease network

"SAveRUNNER Algorithm"). SAveRUNNER provided as output the drug-disease network, where nodes are FDA-approved drugs and diseases, while a link between them occurs only if a drug is predicted to be repositioned for that disease. Among the drugs predicted by SAveRUNNER as repurposable candidates for ALS, we focused on those whose original medical indications referred to diseases connected to ALS in the human disease network.

SAveRUNNER Algorithm

Recently, we developed a new network-medicine-based algorithm for drug repurposing called SAveRUNNER [23], with the aim of efficiently screening novel potential indications for currently marketed drugs against diseases of interest, and optimizing the efficacy of putative validation experiments. A detailed description of SAveRUNNER algorithm can be found in reference [23, 24].

Gene Set Enrichment Analysis

In order to test whether the candidate anti-ALS repurposable drugs predicted by SAveRUNNER can counteract the gene expression perturbations caused by ALS pathophenotype (i.e., if they could up-regulate genes down-regulated by the disease or *vice versa*), we performed a gene set enrichment analysis (GSEA). We first collected three gene expression datasets of ALS patients and control samples

available through the GEO public repository. In particular: (i) the expression profiling by high throughput sequencing (corresponding to RPKM-normalized expression values) of induced pluripotent stem cell (iPSC)-derived motor neurons of 4 ALS patients and 4 controls samples (GSE52202 [33]); (ii) the expression profiling by array (corresponding to normalized expression values obtained from Affymetrix GeneChip 3.1 software) of postmortem spinal cord grey matter from 7 ALS patients and 4 control samples (GSE833 [34]); and (iii) expression profiling by array (corresponding to normalized expression values obtained by using Rosetta error models [35]) of an extensive cohort of well-characterized postmortem central nervous system tissues from 10 ALS patients and 10 control samples (GSE26927 [35]). For the GSE833 dataset, the probe-sets were mapped to official gene symbols using the platform GPL80 (Affymetrix Human Full Length HuGeneFL Array) available from the GEO repository. Multiple probe measurements of a given gene were collapsed into a single gene measurement, by considering the mean. For each dataset, data were processed by applying a logarithmic (log2) transformation of the expression values, and by conducting a preprocessing analysis via the computation of the interquartile range (IQR) for each gene. IQR is a measure of data variability around the median, that is equal to the difference between the 75th and 25th percentiles of the data distribution. Those genes with an IQR value smaller than the 10th percentile of the IQR distribution (corresponding to those genes less scattered around the median) were filtered out. Then, we performed the nonparametric Wilcoxon signed rank test for GSE52202 and GSE26927 datasets with paired samples, and the Mann–Whitney test for GSE833 dataset with unpaired samples. Finally, we adjusted the obtained p values for multiple hypothesis testing, by using Benjamini–Hochberg procedure. In order to select statistically significant differentially expressed genes, we set a threshold of 0.05 on the adjusted p values for the dataset with the largest number of samples (i.e., GSE26927). For the other two datasets (i.e., GSE52202 and GSE833), we obtained adjusted p values that have been always greater than the standard significant level. Thus, only for these datasets, we decided to discard the adjustment of the p values, and to compensate for this shortcoming by using a more severe threshold of 0.01 on the original p values. We used the so-defined three lists of differentially expressed genes as three separated ALS signatures.

Then, we queried the connectivity map (CMap) database that collects high-throughput reduced representation gene expression data obtained by using L1000 assay [36, 37]. The L1000 profiling is performed in a variety of drug-treated human cell lines for which there are well-established culture and treatment protocols. Thus, the CMap database of cellular signatures catalogs transcriptional responses of human cells to chemical and genetic perturbation. A total of 27,927 perturbagens have been profiled in a core set of 9 cell lines to produce 476,251 expression signatures. We used the differentially expressed genes of drug-treated human cell lines from the CMap database as drug signatures.

For each drug that was in both the CMap database and predicted by SAveRUNNER to be effective against ALS, we evaluated the treatment effects on differentially expressed genes that are hallmarks for ALS disease phenotype, by exploiting the CMap query tool for each ALS signature given as a separated input list [36]. The disease signatures and the drug signatures were ordered by increasing fold-change, and then CMap computed an enrichment score (ES) that measures if the effect of the drug could counteract the effect of the disease ($ES < 0$), or not ($ES > 0$) [37, 38]. The idea behind this is the following: one ordered disease signature is compared to one ordered drug signature, to determine whether the highest up-regulated (down-regulated) gene in the disease signature is near the bottom (top) of the drug signature. This would mean that drug and disease have complementary expression profiles ($ES < 0$), and the drug might be a possible treatment option for the disease of interest. Details on the computation of this score are provided in [37–39]. In particular, a selected repurposing candidate drug was considered to have a potential treatment effect against ALS if the drug signature was negatively correlated with the ALS signature. We stated that drugs and diseases were negatively correlated if the corresponding ES was negative, and we assigned a score equal to 1 to that drug for that disease signature. Inspired by the procedure adopted in [20], the number of ALS signature datasets satisfying this criterion was used for each drug as the final GSEA score, which ranges from 0 to N, being N the total number of ALS signatures used. By considering in this study $N = 3$ disease signatures, the maximum GSEA score for each drug will be 3.

Module Significance

As well-established by Network Medicine principles [13, 14, 19], disease-associated genes have unique, quantifiable characteristics that distinguish them from other genes. This observation can be translated into the verification that disease-associated genes do not map randomly in the interactome but, rather, they agglomerate in locally dense and topologically well-defined regions of this network (called *disease modules*), whose nodes show an increased tendency to interact with each other, more frequently than expected by chance.

We investigated whether genes associated to ALS (as well as to other analyzed disorders) had the propensity to aggregate in a local neighborhood of the human interactome, and constituted a statistically significant disease module. To do that, we mapped the disease-associated genes onto the human interactome, we extracted the corresponding disease

subnetwork, and we computed the following three metrics [17]: (i) the total number of interactions (edges); (ii) the size of the largest connected component (LCC); and (iii) the number of edges in the LCC. Then, we complemented these metrics with a measure of statistical significance, named *module significance*, which measures the probability that a given list of disease genes is localized within a certain network neighborhood, more frequently than expected by chance [17]. Specifically, for each analyzed disease, we randomly selected groups of proteins of the same size and degree distribution as the original list of disease genes in the human interactome. We then extracted the corresponding random subnetwork, and we computed the three above-described metrics. This procedure was repeated 1000 times. As reported in the majority of state-of-the-art approaches [40–48], 1000 permutations are commonly used for estimating the power of a randomization test and are considered reasonable for a test at the 5% level of significance. Finally, we derived three distributions for all three metrics corresponding to the subnetwork induced by the random gene set. By using a z-score, we have normalized the three metrics calculated for the original list of disease genes, with respect to the corresponding reference random distribution, and we assigned the p value for the given z statistic. If all three metrics are statistically significant (p value ≤ 0.05), we can conclude that disease genes form statistically significant modules in the human interactome.

Random Walk with Restart Algorithm

We implemented a Random Walk with Restart (RWR) algorithm to measure the closeness between the ALS disease module and the other disease modules in the human interactome network. RWR is an algorithm based on an intuitive concept that revolves around random walks. Given a random walker starting from a given node x, there are two different options at each iteration: either moving to one of its neighboring nodes, or returning to x with a certain probability. Thus, the task is to calculate the most likely locations where the walker is going to be. Formally, the RWR algorithm can be described by the following equation:

$$R_t = \gamma W R_{t-1} + (1-\gamma)E$$

where W is the network adjacency matrix, representing the matrix of transitions between nodes, whose element $W[i,j]$ denotes the transition probability of going from node j to node i; E is the starting point vector, whose element $E[i]$ is equal to 1, if i is a starting node, 0 otherwise; R_t is a probabilities vector, whose element $R_t[i]$ denotes the probability of being at node i at iteration t; γ is a number ranging in (0,1), and $(1-\gamma)$ expresses the probability of "restarting" from the starting point node at each iteration. At iteration $t=0$, the value of R_{t-1} is equal to E. The probabilities vector R_t will be iteratively calculated until the point of convergence is reached (i.e., $R_t = R_{t-1}$, or the difference between probability to stay and the probability to move on is lower than a given threshold). Finally, the RWR returns as output the vector R of the steady-state probabilities for each node in the network.

We run the RWR by considering the adjacency matrix W^{mxm} built from the human interactome as a transition matrix, and the vector E of the ALS disease genes as starting point vector. We selected only those diseases with a frequency to be reached greater than 30 (i.e., number of reached disease genes greater than 30). For each disease, we averaged the RWR steady-state probabilities of the corresponding disease genes and obtained a mean probability for each disease, that is the probability to reach it, when starting from ALS. This disease probability was then normalized by using the modified z-score defined as:

$$z_{mod} = c \cdot \frac{x - \hat{x}}{MAD}$$

where x is the disease probability, $\hat{x}$ is the median value of the distribution of all the disease probabilities, MAD is the median absolute deviation, defined as the median of the absolute difference of the observation from the sample median (i.e., median$(|x-\hat{x}|)$, and c is a scale factor equal to 0.6745, such that z_{mod} is equal to the standard z-score for normal distribution [49]. We named this z-normalized disease probability as *ALS closeness*, and we assigned it the p value corresponding to the z-score. Values of ALS closeness > 2.5 can be labeled as potential positive outliers, whereas values < -2.5 as negative outliers. Diseases corresponding to positive outliers are more likely to be reached by the random walker starting from ALS, and thus represent diseases closer to ALS.

Module Separation

We computed the non-Euclidean separation distance of the ALS disease module with respect to other disease modules as follows [50]:

$$s(A,B) = p_{AB} - \frac{p_{AA} + p_{BB}}{2}$$

where $p(A,B)$ is the network proximity defined as:

$$p(A,B) = \frac{1}{|A| + |B|}\left[\sum_{a \epsilon A} \min_{b \epsilon B} d(a,b) + \sum_{b \epsilon B} \min_{a \epsilon A} d(b,a)\right]$$

and $d(a,b)$ is the shortest distance between disease gene a of module A, and disease gene b of module B. A positive value

for the separation measure indicates that two disease modules are topologically well separated in the human interactome, whereas a negative value for the separation measure indicates that two disease modules are located in the same network neighborhood, and thus they overlap. To evaluate the significance of module separation across two disease-specific modules (A, B) of disease genes, we built a reference distance distribution corresponding to the expected distance between two randomly selected groups of proteins of the same size and degree distribution as the original two sets of disease genes in the human interactome. The random selection was repeated 1000 times in order to build the reference distance distribution. The module separation measure across the two lists of disease genes was z-score-normalized by using the mean and the standard deviation of the reference distribution. Subsequently, the p value for the given z statistic was calculated. A p value <0.05 indicates that the module separation in the human interactome of the two lists of disease genes is more (or less, see below) than expected by chance.

Pathways Enrichment Analysis

In order to investigate the pathways in which the target genes of the most promising histamine-related compounds (i.e., amoxapine, clomipramine, mianserin, and modafinil) are involved with, we queried the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database [51] and the Reactome pathway knowledgebase [52], by using the Enrichr web tool [53]. The p values were adjusted with the Benjamini–Hochberg method, and a threshold equal to 0.05 was set to identify the molecular pathways significantly enriched among the target genes given as input lists.

Results

ALS-drug Network

In the present study, we applied the recently developed SAveRUNNER algorithm [23, 24], to identify repurposable drug candidates for ALS.

SAveRUNNER requires a list of drug targets as input, and a list of disease genes to evaluate the extent to which a given drug can be eventually repositioned to treat a disease.

Here, the disease-associated genes were downloaded from Phenopedia [30], which provides 267 ALS-associated genes, whereas drug-target associations were obtained from DrugBank [31]. In particular, we assembled target information about a total of 1860 FDA-approved drugs. Besides considering single drugs in our input list of drug targets, we considered also 80 drug combinations that are lately gaining more interest in ALS research. The complete list of the 1940 analyzed drugs is provided as Supplementary Table 1 along with the corresponding number of target proteins, for a total of 2138 unique target proteins.

The rationale behind SAveRUNNER lies in the hypothesis that, for a drug to be effective against a specific disease, its associated targets (drug module) and the disease-specific associated genes (disease module) should be nearby in the human interactome [21]. To quantify the vicinity between drug and disease modules, SAveRUNNER implements a novel network similarity measure and assesses its statistical significance by applying a degree-preserving randomization procedure [23].

Its novelty resides in implementing a procedure to prioritize the predicted off-label drug indications for a given disease. This prioritization procedure exploits a clustering analysis to reward associations between drugs and diseases belonging to the same cluster, based on the assumption that if a drug and a disease group together, most likely that drug can be effectively repurposed for that disease. In this sense, we say that drugs and diseases that are members of the same group are more similar to each other than to members of other groups.

As output, SAveRUNNER releases a weighted bipartite drug-disease network, where a link between a drug and a disease occurs if the corresponding drug targets and disease genes are closer in the interactome than expected by chance. The weight of their interaction corresponds to the network-based similarity measure.

In this study, SAveRUNNER identified 403 repurposable drugs (out of 1940 drugs) that were significantly associated

Table 1 The table lists histamine receptor modulators (with corresponding Drug Bank number) that are identified by SAveRUNNER as repurposable drugs for ALS. The action of each ligand on H1R, H2R, H3R, and H4R receptors is indicated, together with the detail of their additional receptor ligand binding properties (as from https://www.drugbank.ca/drugs/)

Drug Bank code	H1R	H2R	H3R	H4R	Other receptor	p value	Adjusted similarity	GSEA score	Ref
Amoxapine DB00543	Antagonist	—	—	Agonist	D1-3, M1, 5-HT1-3,6,7	0.006	0.89	1	[59, 60]
Clomipramine DB01242	Antagonist/inverse agonist	—	—	—	5-HT2	0.003	0.99	2	[61]
Mianserin DB06148	Antagonist/inverse agonist	—	—	Agonist	D1-3α1,2, 5-HT1, 2,6,7,	0.06	0.92	1	[62]

(p value < 0.07) with ALS (Supplementary Table 2). Among those, we found modafinil (adjusted similarity value = 0.99, p value = 0.04), a drug elevating histamine release and levels in the neocortex and hypothalamus [54–56], whose effects are abolished by depletion of neuronal histamine [57], and that is used to treat hypersomnolence of narcolepsy and to increase locomotor activity. Modafinil significantly affects also the dopamine transporter, acting as a dopamine reuptake inhibitor, moreover activates glutamatergic and inhibits GABAergic circuits [58]. Corroborating the histaminergic implication of modafinil with ALS, SAveRUNNER identified additional drugs mainly (but not exclusively) interfering with the histaminergic system, such as amoxapine [59, 60] (adjusted similarity = 0.89, p value = 0.006), clomipramine [61] (adjusted similarity = 0.99, p value = 0.003), and mianserin [62] (adjusted similarity = 0.92, p value = 0.06) (Fig. 2, Table 1, and Supplementary Table 2). Furthermore, by searching for drug combinations and analyzing compounds already adopted in clinical trials for ALS such as arimoclomol, erythropoietin, masitinib, minocycline, ozanezumab, and perampanel, SAveRUNNER disclosed that modafinil indeed improves the p value and adjusted similarity ranking of erythropoietin (adjusted similarity = 0.99, p value = 0.05), masitinib (adjusted similarity = 0.99, p value = 0.04), and

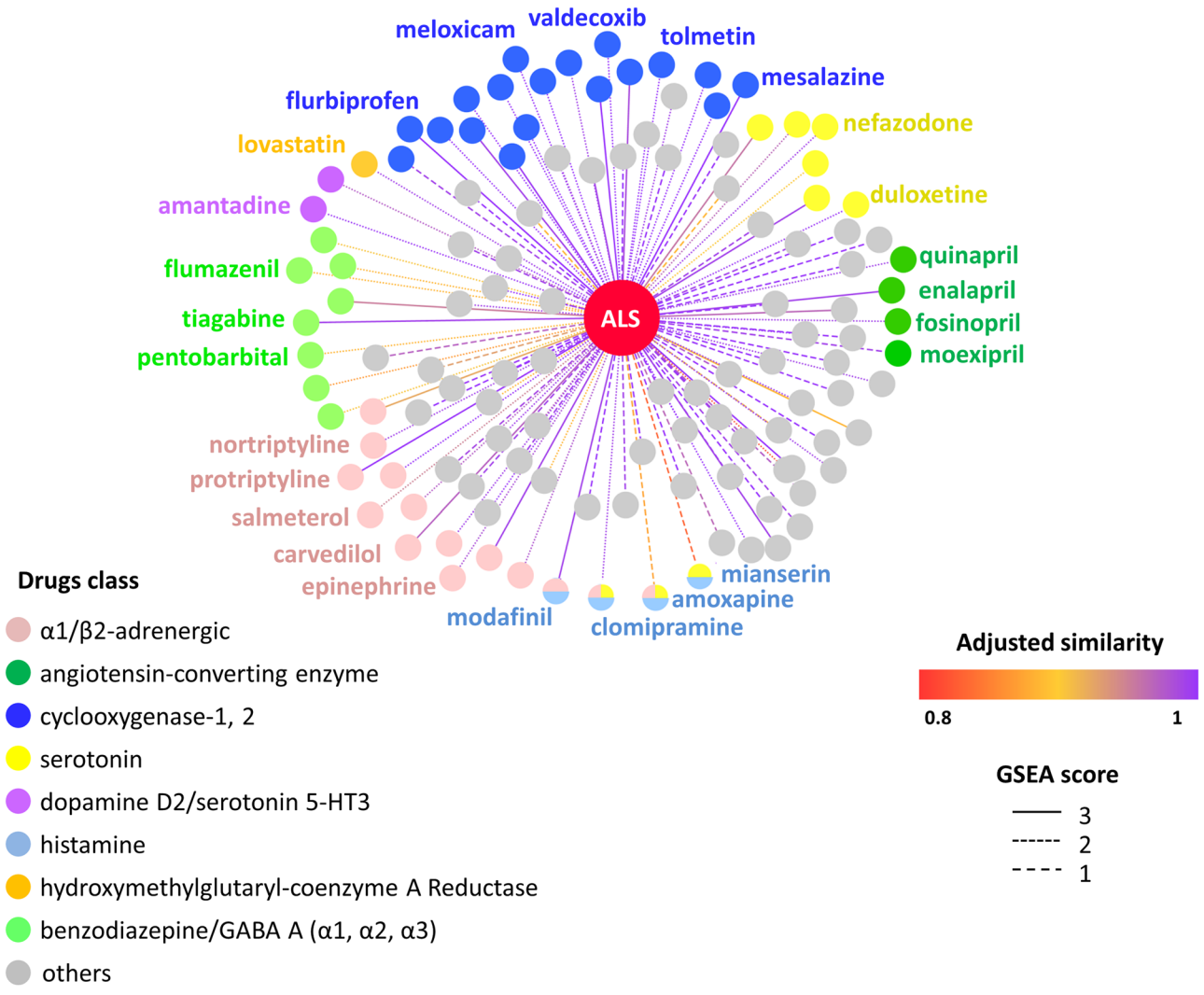

Fig. 2 Schematic representation of the predicted drug-disease ALS network. This sketch shows the high-confidence predicted drug-disease associations (p value < 0.07) connecting ALS with 121 FDA-approved non-ALS drugs showing a GSEA score > 0. Drugs are colored according to their targeting of receptor/enzyme classes reported in the legend. The edge color indicates the adjusted similarity increasing from red to violet. The different edge line types correspond to different GSEA scores ranging from 1 (dashed lines) to 3 (solid line). Note that the p value threshold of 0.07 allowed us to include some promising histaminergic compounds such as mianserin, showing a borderline p value of 0.06

perampanel (adjusted similarity = 0.99, p value = 0.05) (Supplementary Table 2).

GSEA Analysis of Anti-ALS Repurposable Drugs

In order to further investigate the anti-ALS repurposable drugs predicted by SAveRUNNER, we performed a GSEA, by using the transcriptome data from nervous system tissues of ALS patients as disease signatures, and the gene expression data of drug-treated human cell lines from the Connectivity Map (CMap) database as drug signatures. For each drug predicted by SAveRUNNER and included in the CMap database, we calculated a GSEA score as an indication of its possible counteraction to the gene expression perturbations caused by ALS pathophenotype. In particular, for each ALS dataset, we selected drugs whose signatures were negatively correlated with the ALS signature according to the CMap query tool [37–39], as able to have a potential treatment effect against genes that are a hallmark of ALS phenotype (see "Methods"). The assigned GSEA score, ranging from 0 to 3, corresponded to the number of ALS datasets satisfying this criterion for a specific drug.

The GSEA analysis highlighted a total of 121 out of the 403 candidate drugs to be repositioned against ALS, including 21 with a GSEA score of 3, 52 with a GSEA score equal to 2, and 48 with a GSEA score equal to 1 (Fig. 2, Supplementary Table 2).

Drugs showing a GSEA score > 0 belong to several interesting classes, some directly involved in the mechanisms and pathways of ALS, including again histamine receptor modulators, angiotensin-converting enzyme (ACE) inhibitors, Prostaglandin G/H synthase 1 and 2 (cyclooxygenase 1/2 isozymes) inhibitors, α1/β2-adrenergic receptors, dopamine D2 receptors, and benzodiazepine/GABA receptor modulators (Fig. 2, Supplementary Table 2). Among the histamine modulators, it is important to highlight that modafinil indeed showed the highest GSEA score equal to 3. Remarkably, also the other histamine receptor modulators (i.e., amoxapine, clomipramine, and mianserin) were confirmed by GSEA analysis, showing GSEA scores > 0 (Table 1).

Prediction of ALS Comorbidity

In order to predict potential comorbidity patterns between ALS and other diseases, we implemented two network-based approaches that measure the comorbidity: (1) the *Random Walk with Restart* (RWR) algorithm; (2) the *module separation* measure.

Once verified that ALS-associated genes formed a statistically significant disease module in the human interactome network (Supplementary Table 3), we applied the RWR algorithm, which computed the closeness between ALS disease module (starting points) and other diseases in the human interactome, by assigning to each disease a modified z-score normalized value, defined as *ALS closeness* score (see "Methods"). Values of this score greater than 2.5 were considered as positive outliers and therefore diseases closer to ALS. Among them, we interestingly found frontotemporal dementia (modified z-score = 6.65), vascular dementia (modified z-score = 5.21), muscle weakness (modified z-score = 5.18), neuromuscular diseases (modified z-score = 3.27), polyneuropathies (modified z-score = 2.89), and diabetic neuropathies (modified z-score = 2.58), pathologies that all share some features with ALS, although within different severity degrees, comorbidities and pharmacoepidemiology, and for which some of the anti-ALS drugs predicted by SAveRUNNER/GSEA (i.e., clomipramine, mianserin) were originally approved (Fig. 3a, Supplementary Table 3).

These results appeared to be confirmed also by computing the module separation between the ALS module and the other analyzed diseases (Fig. 3b, c). At first, we ensured that all the analyzed diseases (i.e., neuromuscular diseases, muscle weakness, frontotemporal dementia, polyneuropathies, vascular dementia, vascular, and diabetic neuropathies) formed statistically significant disease modules in the human interactome network (Supplementary Table 3). Then, we found that the ALS disease module directly overlapped with the above-mentioned disease modules in a statistically significant way (Fig. 3b), showing negative separation values and p values ≤ 0.05 (Fig. 3c, Supplementary Table 3), and thus corroborating that a potential ALS treatment can be derived from the arsenal of therapies approved for other specific diseases.

Pathways Analysis of Histamine-related Compounds

Seeking to probe if the most promising histaminergic compounds pointed out by our analysis (i.e., amoxapine, clomipramine, mianserin, and modafinil) could act in the ALS disease pathway, we next investigated the pathways in which their target genes are involved, by performing a functional enrichment analysis via the Enrichr web tool [53]. Of note, the significantly enriched pathways comprised, for instance, "amine ligand-binding receptors, class A/1 (rhodopsin-like) receptors, GPCR ligand binding, GPCR downstream signaling, G alpha (i)/G alpha (q)/G alpha (s) signaling events, CREB signaling pathway via PKC and MAPK, amine compound SLC transporters" that include the signal transduction machinery of histamine receptor activation and pathways that are known to be deregulated during ALS (Fig. 4 and Supplementary Table 4).

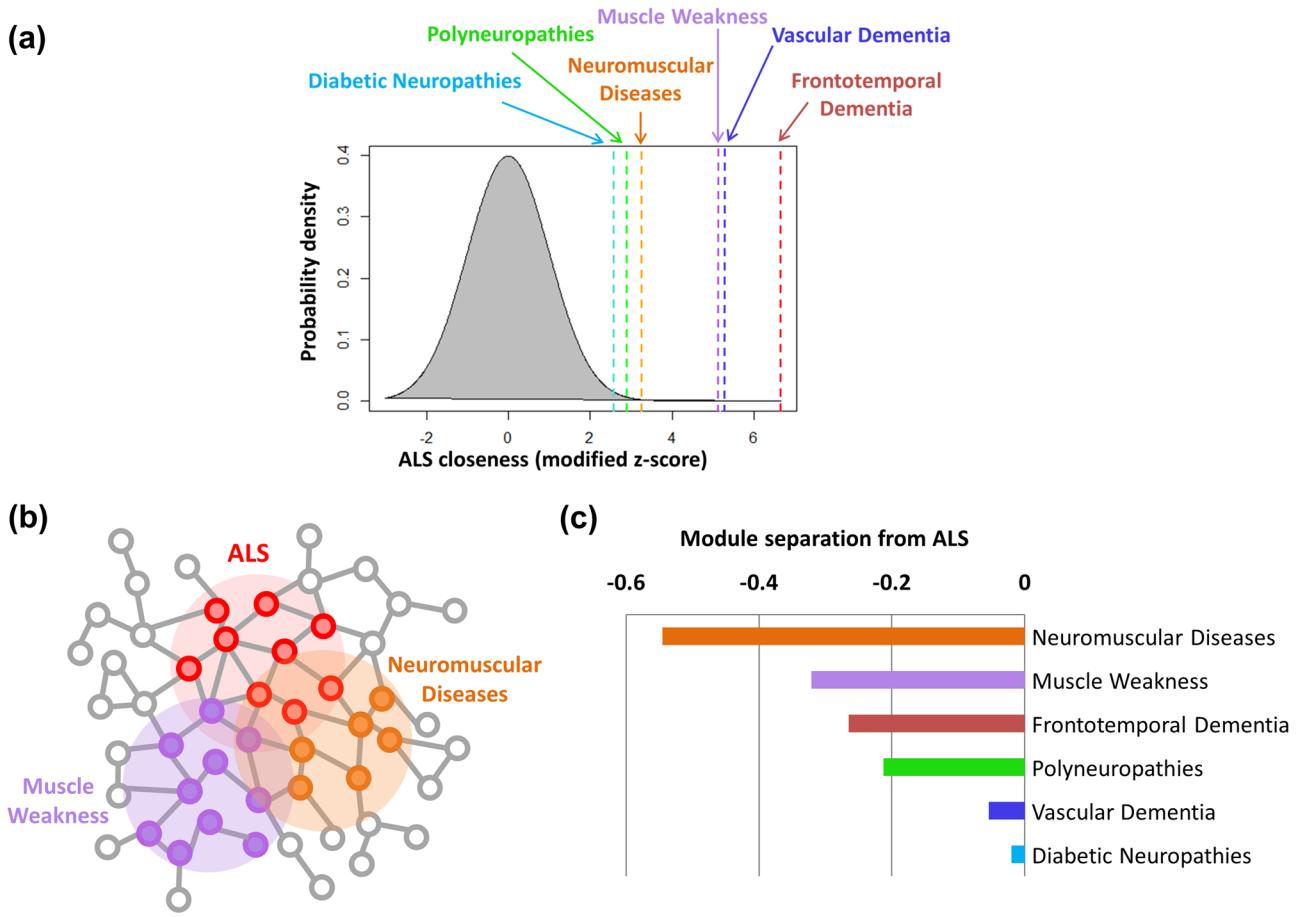

Fig. 3 Network-based modules analysis. **(a)** Distribution of modified z-score normalized probabilities (*ALS closeness*) of nodes that are visited by the Random Walk with Restart (RWR) algorithm starting from nodes belonging to the Amyotrophic Lateral Sclerosis (ALS) disease module. The RWR probabilities of all visited nodes belonging to a disease are averaged and z-score normalized by using a modified z-score (z_{mod}). Values of $z_{mod} > 2.5$ can be labeled as potential positive outliers, while values of $z_{mod} < -2.5$ as negative outliers. Diseases corresponding to positive outliers are more likely to be reached by the random walker starting from ALS, and thus represent diseases closer to ALS. The *ALS closeness* of frontotemporal dementia, vascular dementia, muscle weakness, neuromuscular diseases, polyneuropathies, and diabetic neuropathies are highlighted with red, blue, violet, orange, green, light blue dashed lines, respectively. **(b)** Sketch of the overlapping modules identified by disease genes of ALS and other diseases in the human interactome. **(c)** Bar plot reporting the values of the module separation measure computed between ALS and the other analized disease modules

Discussion

New technologies have shaped an outburst of results about CNS diseases comprising ALS at an unprecedented speed. Innovative computational approaches, among which scalable algorithmic methodologies, are now being established and implemented to address questions linked to human health and diseases, by incorporating different data types such as omics data, digitalized medical records, whole-genome sequences, and phenotypes signatures [63]. Here, we successfully applied a recently developed network medicine-based algorithm, called SAveRUNNER, to identify candidate drugs repurposable for ALS. Our study was motivated by the ultimate goal of linking experimental knowledge with computational analysis, and prospectively translating this information into the clinic. By prioritizing network-predicted drugs by decreasing values of their network similarity with ALS, in addition to catecholamine, dopamine, serotonin, histamine, and GABA receptor modulators, as well as angiotensin-converting enzymes, cyclooxygenase isozymes, and serotonin transporter inhibitors, we found some interesting no customary ALS drugs such as modafinil (2-[(diphenylmethyl) sulfinyl] acetamide) (p value ≤ 0.05, GSEA score $= 3$). The compound was developed a long time ago as a potent, long-lasting wake-promoting substance and was approved in 1998 by FDA (Provigil®) for the treatment of sleep disorders [64]. Modafinil normalizes cognitive functions in sleep-deprived conditions, and being non-addictive has been clinically investigated for nicotine and cocaine addiction, moreover for attention deficit, affective disorders,

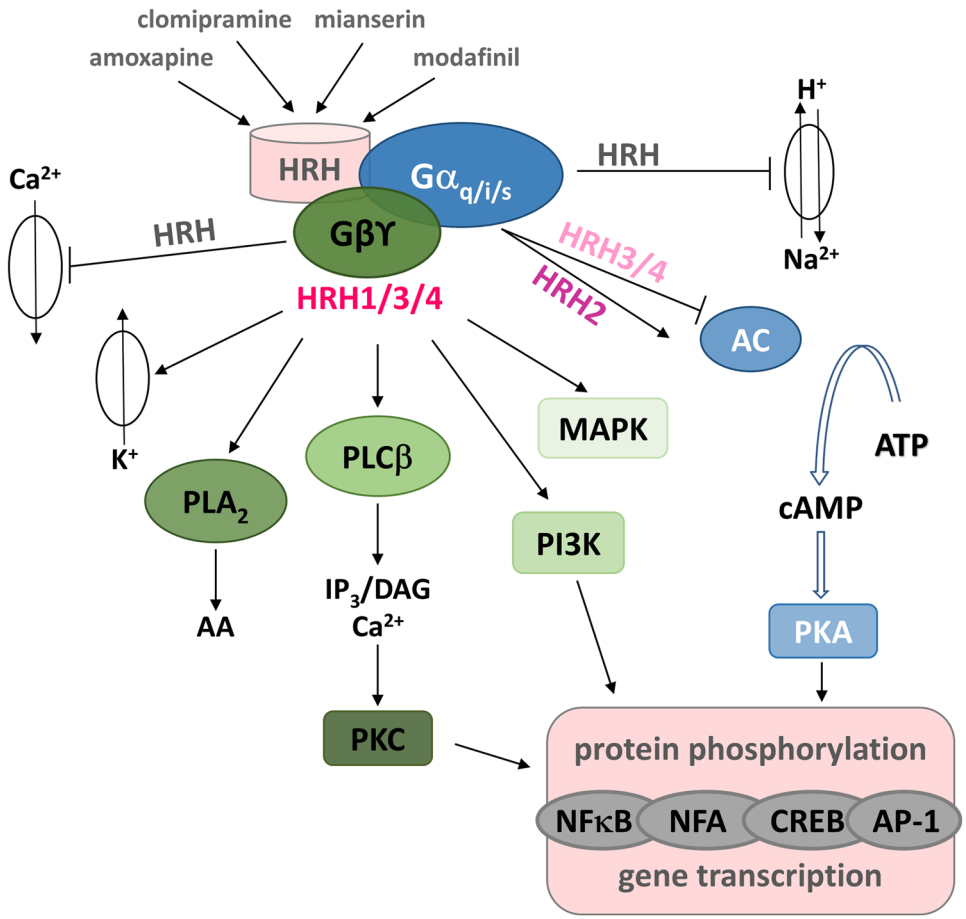

Fig. 4 Histamine receptor signaling cascades that are deregulated during ALS pathological conditions. Signaling cascades driven by amoxapine, clomipramine, mianserin, and modafinil binding to G protein-coupled histamine receptors (HRH), comprise pathways that are deregulated during ALS pathological conditions (MAPK, PLC, PLA2, PI3K, PKC, PKA, NFκB, NFA, CREB, AP-1). Remarkably, these same pathways emerged as significantly enriched amongst the target genes of the histaminergic compounds given as input of the Enirchr web tool (see Supplementary Table 4). AA arachidonic acid, AC adenylate cyclase, AP-1 activator protein 1, cAMP cyclic AMP, CREB cAMP response element-binding protein, DAG diacyl glycerol, HRH histamine receptor, IP3 inositol 3-phosphate, MAPK microtubule-associated protein kinase, PI3K phosphoinositide 3-kinase, PKA cAMP-dependent protein kinase, PKC protein kinase C, PLA_2 phospholipase A2, PLCβ phospholipase C beta, NFA nuclear factor 1 associate, NFκB nuclear factor kappa-light-chain-enhancer of activated B cells

depressions, and schizophrenia, while preclinical evidence suggests effects also in Alzheimer's and Parkinson's diseases [65–68]. A further assessment indicates that modafinil leads to a slight improvement of fatigue syndrome in chronic neurological disorders [69], myotonic dystrophy type 2 [70], and surprisingly also ALS [71], thus confirming and validating the prioritization of modafinil that emerged from our SAveRUNNER/GSEA drug-disease network predictive results.

Although the brain areas where modafinil exactly operates are proven difficult to localize [72], primary targets include the following: subcortical thalamus, hypothalamus and amygdala, for reinforcing activation and maintenance of wakefulness and cognitive performance [73]; thalamocortical circuits, for increasing electrical coupling among cortical interneurons [74]; visual, frontal cortex and cerebellum, for increasing whole functional connectivity [75]; finally the ventromedial region of the spinal cord, for normalizing hyperreflexia [76]. Because the frontal and motor cortex with the ventral spinal cord enclosing motor neurons show distinct patterns of neurodegeneration in ALS, and moreover diffuse thalamic and cerebellar abnormalities are also present in ALS patients [77, 78], modafinil targeting these same brain areas would thus support our SAveRUNNER/GSEA predictive results for ALS and encourage its exploitation and repurposing.

Most research on modafinil's wake-promoting actions has highlighted monoaminergic effects through stimulation of histamine, norepinephrine, serotonin, dopamine, and orexin systems in the brain. Besides working as a dopamine reuptake inhibitor [58], modafinil indirectly activates

the histaminergic system [72], presumably via attenuation of the inhibitory GABAergic input to histaminergic neurons located in the tuberomamillary nucleus of the posterior hypothalamus, and through intensification of the histaminergic tone through orexinergic neurons [55, 72, 79, 80]. Although the way modafinil functions at the circuits' level is still unclear, the possibility that it might act on ALS through a modulation of the histaminergic circuit is substantiated by our SAveRUNNER/GSEA analysis showing that several additional histaminergic modulators such as amoxapine, clomipramine, and mianserin are indeed significantly associated with ALS, by possessing a GSEA score greater than 0. Of note, the SAveRUNNER/GSEA histaminergic predictions find a solid experimental support by preclinical results obtained in the best characterized animal model for ALS, the SOD1-G93A mouse [81], where the histamine precursor histidine was proven to ameliorate pathological features of ALS, delay disease progression, improve motor performance, increase lifespan, decrease motor neuron loss and neuroinflammation in the spinal cord, finally reduce neuromuscular junction fragmentation and muscle atrophy [82]. Actually, the histamine compounds are not new as drug targets in various animal models of diseases, and are moreover suggested to show a clear value in a wide range of clinical CNS conditions [83] comprising ALS [84, 85].

Contemporary approaches to human disease classification are in part still grounded on observational correlations between pathological analysis and existing information about clinical conditions, often neglecting the interrelated features of many diseases, because of a reductionist paradigm that has guided clinical diagnosis in the old days. However, there is now mounting attention paid to disease classifications that rely on Network Medicine information and data correlation. In the present work, by adopting a network-based module separation measure and an RWR algorithm, we gained an additional probative degree of the prospective use of histaminergic compounds in ALS. In particular, we demonstrated comorbidity patterns and disease module overlaps between ALS and pathologies including dementia and polyneuropathies that are already alleviated in clinical practice by histamine receptor modulators such as mianserin and clomipramine. In this context, ALS can be viewed as the breakdown of a sequence of linked networks that integrate the primary disease-causing genes with the disease-modifying genes shared by all diseases, with their network-based environmental and behavioural determinants, including those that control gene expression at the transcriptional or epigenetic level, and those that drive posttranslational modifications of the proteome or uniqueness of the metabolome. These subnetwork determinants are those that contribute to yield comorbidities between complex illness such as dementia and ALS on one side, as evinced from our analysis, but on the other to distinguish each clinical phenotype in highly individualized ways. By further exploring the interplay between drug/disease networks and ALS, we are confident to be able to more comprehensively and accurately map this disease, thus facilitating the emergence of more favourable therapeutic solutions.

In summary, there are three overarching findings in our study. First, SAveRUNNER/GSEA predictive analysis supports our previous hypothesis corroborated by preclinical evidence that some histaminergic compounds might have a successful translational impact in the development of therapeutics to be repositioned for ALS. Second, among various histaminergic modulators, our analysis prioritizes modafinil, a drug targeting specific brain regions that in part overlap with those controlling motor circuits well known to be affected in ALS. This notion can also provide a basis for further dissecting the mode of action and target of modafinil, subtyping ALS features and tracking the disease. Finally, by matching network-predictive with previous preclinical testing, our SAveRUNNER/GSEA algorithm introduces a more integrative framework for the identification and future clinical assessment of drugs possibly functioning in ALS.

The strategy of "one-molecule-one-target" for defeating ALS has failed so far and we strongly believe that therapies based on "multi-target drug" and/or "polypharmacology" approaches must be addressed next, for understanding the several heterogeneous aspects and halting the progression of the disease. Also in this regard, modafinil seems to be a very promising candidate. Not only it can interfere with histaminergic, dopaminergic, glutamatergic and GABAergic neurotransmission, but modafinil is identified by SAveRUNNER as a drug that combined with compounds actually in a clinical trial for ALS such as masitinib [86], erythropoietin [87], and perampanel [88] significantly improves their p-value and adjusted similarity ranking. Although the histaminergic drugs certainly meet some of the requirements for being possibly repurposed in ALS, validation of druggable targets has to be continuous and further research about histamine signaling, its dosing and druggability, is certainly needed to deepen our understanding of the insurgence, progression and pharmacological treatment of ALS. As a predictive value matures and scales up, as shown in the present work by our SAveRUNNER/GSEA and module separation analysis, continuous integration is also needed: this is our ongoing and future aim.

Indeed, we believe that the network-medicine approach to drug repositioning, as implemented by SAveRUNNER/GSEA analysis, can significantly catalyze innovation in the discovery of promising repurposable drug candidates that deserve further investigation and experimental validation for ALS. However, the first step in exploring the interplay between networks and human diseases is to assess how comprehensive and accurate the current molecular and phenotypic network maps are for humans. The past few years

have witnessed systematic efforts to increase the coverage of human interactome maps, estimate the interactome size and correct for known biases. Still, human interactome maps remain incomplete and noisy, a fact that needs to be taken into account when studying diseases. Yet, the list of ALS-associated genes is far from completeness and continuously updated by the discovery of mutated genes having a phenotypic impact on the disease. Thus, suffering from the notorious incompleteness of literature-based input parameters, SAveRUNNER may also lead to incomplete predictions. In this regard, a first example might be clemastine, a direct histamine H1 receptor antagonist that was shown to ameliorate ALS disease progression in the SOD1-G93A mouse model [89, 90], but that is not included in the candidate drugs predicted by SAveRUNNER algorithm. Satisfying the histaminergic hypothesis, on the other hand SAveRUNNER pointed out an unconventional histamine receptor modulator, modafinil (Provigil®), which elevates histamine release and levels in the neocortex and hypothalamus [54, 55, 91]. Allowing to greatly save on time and resources, SAveRUNNER has perhaps identified a promising and worth of investigation candidate that a conventional drug discovery preclinical study would not have considered for ALS.

These considerations confirm the impelling need to find further synergies between network-based analysis and preclinical drug testing, in order to draw deeper knowledge of the inherent complexity of ALS and, more importantly, develop a cure for the disease. Despite all attempts to search for treatments have failed so far, and current therapies can only reduce morbidity [10], we believe that the right answer to ALS might come from a multidrug strategy or broad-spectrum molecules capable of interfering with multiple pathological pathways, as modafinil and histaminergic compounds might indeed be doing. Only further search will tell us what is exactly going to improve the therapeutic development in ALS.

Abbreviations ALS: Amyotrophic lateral sclerosis; CMap: Connectivity map; FDA: Food and Drug Administration; GSEA: Gene set enrichment analysis; KEGG: Kyoto Encyclopedia of Genes and Genomes; RWR: Random Walk with Restart; SAveRUNNER: Searching offlAbel dRUg aNd NEtwoRk

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13311-021-01064-z.

Required Author Forms Disclosure forms provided by the authors are available with the online version of this article.

Author contribution PP and CV contributed to conceptualization, design, funding acquisition and supervision of the study; GF and FC performed computational analyses; CV and SA performed biological analysis, interpretation, and elaboration of biological results. All authors participated to the manuscript draft and editing.

Funding This work was financially supported by PRIN 2017—Settore ERC LS2—Codice Progetto 20178L3P38 and by The Italian Ministry of Health (Ricerca Corrente) to Fondazione Santa Lucia IRCCS.

Data availability All data generated during this study are included in this published article as supplementary material. SAveRUNNER code is open-source and available at https://github.com/sportingCode/SAveRUNNER, together with an exhaustive and well-documented user guide, which includes a detailed description of all R scripts and all input/output files through a working example of SAveRUNNER application on 15 diseases.

Declarations

Conflict of interest The authors declare no competing interests.

References

1. Masrori P, Damme PV. Amyotrophic lateral sclerosis: a clinical review. Eur J Neurol 2020; 0: 1–12.
2. Oskarsson B, Gendron TF, Staff NP. Amyotrophic Lateral Sclerosis: An Update for 2018. Mayo Clin Proc 2018; 93: 1617–1628.
3. Gromicho M, Figueiral M, Uysal H, et al. Spreading in ALS: The relative impact of upper and lower motor neuron involvement. Ann Clin Transl Neurol 2020; 7: 1181–1192.
4. Burk K, Pasterkamp RJ. Disrupted neuronal trafficking in amyotrophic lateral sclerosis. Acta Neuropathol (Berl) 2019; 137: 859–877.
5. Ravits J, Paul CJP. Focality of upper and lower motor neuron degeneration at the clinical onset of ALS. Neurology 2007; 68: 1571–1575.
6. Cappello V, Francolini M. Neuromuscular Junction Dismantling in Amyotrophic Lateral Sclerosis. Int J Mol Sci 2017; 18 (10): 2092.
7. Guo Luo JZ, Jianxun Yi, Changling Ma, et al. Defective Mitochondrial Dynamics Is an Early Event in Skeletal Muscle of an Amyotrophic Lateral Sclerosis Mouse Model. PLoS ONE 2013; 8: e82112.
8. Beers DR, Appel SH. Immune dysregulation in amyotrophic lateral sclerosis: mechanisms and emerging therapies. Lancet Neurol 2019; 18: 211–220.
9. Verber N, Shaw PJ. Biomarkers in amyotrophic lateral sclerosis: a review of new developments. Curr Opin Neurol 2020; 33: 662–668.
10. Wobst HJ, Mack KL, Brown DG, et al. The clinical trial landscape in amyotrophic lateral sclerosis—Past, present, and future. Med Res Rev 2020; 40: 1352–1384.
11. Pushpakom S, Iorio F, Eyers PA, et al. Drug repurposing: progress, challenges and recommendations. Nat Rev Drug Discov 2019; 18: 41–58.
12. Kukharsky MS, Skvortsova VI, Bachurin SO, et al. In a search for efficient treatment for amyotrophic lateral sclerosis: Old drugs for new approaches. Med Res Rev. Epub ahead of print 20 August 2020. https://doi.org/10.1002/med.21725.
13. Caldera M, Buphamalai P, Müller F, et al. Interactome-based approaches to human disease. Curr Opin Syst Biol 2017; 3: 88–94.
14. Barabási A-L, Gulbahce N, Loscalzo J. Network medicine: a network-based approach to human disease. Nat Rev Genet 2011; 12: 56–68.
15. Barabási A-L. Network Medicine — From Obesity to the "Diseasome". N Engl J Med 2007; 357: 404–407.

16. Silverman EK, Schmidt H, Anastasiadou E, et al. Molecular networks in Network Medicine: Development and applications. WIREs Syst Biol Med 2020; 12: e1489.
17. Paci P, Fiscon G, Conte F, et al. Gene co-expression in the interactome: moving from correlation toward causation via an integrated approach to disease module discovery. Npj Syst Biol Appl 2021; 7: 1–11.
18. Sonawane AR, Weiss ST, Glass K, et al. Network Medicine in the Age of Biomedical Big Data. Front Genet 2019; 10: 294.
19. Goh K-I, Cusick ME, Valle D, et al. The human disease network. Proc Natl Acad Sci 2007; 104: 8685–8690.
20. Zhou Y, Hou Y, Shen J, et al. Network-based drug repurposing for novel coronavirus 2019-nCoV/SARS-CoV-2. Cell Discov 2020; 6: 1–18.
21. Cheng F, Desai RJ, Handy DE, et al. Network-based approach to prediction and population-based validation of in silico drug repurposing. Nat Commun 2018; 9: 2691.
22. Morselli GD, do Valle Í, Zitnik M, Ameli A, Gan X, Varol O, Ghiassian SD, Patten JJ, Davey RA, Loscalzo J, Barabási AL. Network medicine framework for identifying drug-repurposing opportunities for COVID-19. Proc Natl Acad Sci U S A 2021; 118(19):e2025581118.
23. Fiscon G, Conte F, Farina L, et al. SAveRUNNER: A network-based algorithm for drug repurposing and its application to COVID-19. PLOS Comput Biol 2021; 17: e1008686.
24. Fiscon G, Paci P. SAveRUNNER: an R-based tool for drug repurposing. BMC Bioinformatics 2021; 22: 150.
25. Chatr-Aryamontri A, Breitkreutz BJ, Oughtred R, et al. The BioGRID interaction database: 2015 update. Nucleic Acids Res 2015; 43: D470-478.
26. Peri S, Navarro JD, Kristiansen TZ, et al. Human protein reference database as a discovery resource for proteomics. Nucleic Acids Res 2004; 32: D497-501.
27. Licata L, Briganti L, Peluso D, et al. MINT, the molecular interaction database: 2012 update. Nucleic Acids Res 2012; 40: D857-861.
28. Orchard S, Ammari M, Aranda B, et al. The MIntAct project--IntAct as a common curation platform for 11 molecular interaction databases. Nucleic Acids Res 2014; 42: D358-363.
29. Breuer K, Foroushani AK, Laird MR, et al. InnateDB: systems biology of innate immunity and beyond-recent updates and continuing curation. Nucleic Acids Res 2013; 41: D1228-1233.
30. Yu W, Clyne M, Khoury MJ, et al. Phenopedia and Genopedia: disease-centered and gene-centered views of the evolving knowledge of human genetic associations. Bioinformatics 2010; 26: 145–146.
31. Wishart DS, Feunang YD, Guo AC, et al. DrugBank 5.0: a major update to the DrugBank database for 2018. Nucleic Acids Res 2018; 46: D1074.
32. Wang Y, Zhang S, Li F, et al. Therapeutic target database 2020: enriched resource for facilitating research and early development of targeted therapeutics. Nucleic Acids Res 2020; 48: D1031–D1041.
33. Sareen D, O'Rourke JG, Meera P, et al. Targeting RNA foci in iPSC-derived motor neurons from ALS patients with a C9ORF72 repeat expansion. Sci Transl Med 2013; 5: 208ra149.
34. Dangond F, Hwang D, Camelo S, et al. Molecular signature of late-stage human ALS revealed by expression profiling of postmortem spinal cord gray matter. Physiol Genomics 2004; 16: 229–239.
35. Durrenberger PF, Fernando FS, Magliozzi R, et al. Selection of novel reference genes for use in the human central nervous system: a BrainNet Europe Study. Acta Neuropathol (Berl) 2012; 124: 893–903.
36. Subramanian A, Narayan R, Corsello SM, et al. A Next Generation Connectivity Map: L1000 Platform and the First 1,000,000 Profiles. Cell 2017; 171: 1437-1452.e17.
37. Lamb J, Crawford ED, Peck D, et al. The Connectivity Map: using gene-expression signatures to connect small molecules, genes, and disease. Science 2006; 313: 1929–1935.
38. Sirota M, Dudley JT, Kim J, et al. Discovery and preclinical validation of drug indications using compendia of public gene expression data. Sci Transl Med 2011; 3: 96ra77.
39. Subramanian A, Tamayo P, Mootha VK, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U A 2005; 102: 15545–15550.
40. Anderson MJ, Legendre P. An empirical comparison of permutation methods for tests of partial regression coefficients in a linear model. J Stat Comput Simul 1999; 62: 271–303.
41. Hayes AF. Cautions in testing variance equality with randomization tests. J Stat Comput Simul 1997; 59: 25–31.
42. Kennedy PE. Randomization Tests in Econometrics. J Bus Econ Stat 1995; 13: 85–94.
43. Marozzi M. A bi-aspect nonparametric test for the two-sample location problem. Comput Stat Data Anal 2004; 44: 639–648.
44. Shipley B. A permutation procedure for testing the equality of pattern hypotheses across groups involving correlation or covariance matrices. Stat Comput 2000; 10: 253–257.
45. Wan Y, Cohen J, Guerra R. A permutation test for the robust sib-pair linkage method. Ann Hum Genet 1997; 61: 77–85.
46. Smith EP. Randomization methods and the analysis of multivariate ecological data. Environmetrics 1998; 9: 37–51.
47. Bailer AJ. Testing variance equality with randmization tests. J Stat Comput Simul 1989; 31: 1–8.
48. An Almost Exact Solution for the Multivariate Behrens-Fisher Problem - Fortunato Pesarin - Google Libri, https://books.google.it/books?id=qoLFoQEACAAJ&dq=An+Almost+Exact+Solution+for+the+Multivariate+Behrens-Fisher+Problem&hl=it&sa=X&ved=2ahUKEwjPpbrQwPXqAhXE-qQKHcfVD5gQ6AEwAHoECAEQAQ (accessed 30 July 2020).
49. Iglewicz B, Hoaglin D. How to detect and handle outliers. ASQC Basic Ref Qual Control Stat Tech 16.
50. Menche J, Sharma A, Kitsak M, et al. Uncovering disease-disease relationships through the incomplete interactome. Science 2015; 347(6224): 1257601.
51. Kanehisa M, Sato Y, Kawashima M, et al. KEGG as a reference resource for gene and protein annotation. Nucleic Acids Res 2016; 44: D457–D462.
52. Jassal B, Matthews L, Viteri G, et al. The reactome pathway knowledgebase. Nucleic Acids Res 2020; 48: D498–D503.
53. Kuleshov MV, Jones MR, Rouillard AD, et al. Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. Nucleic Acids Res 2016; 44: W90-97.
54. Ishizuka T, Sakamoto Y, Sakurai T, et al. Modafinil increases histamine release in the anterior hypothalamus of rats. Neurosci Lett 2003; 339: 143–146.
55. Ishizuka T, Murotani T, Yamatodani A. Modanifil activates the histaminergic system through the orexinergic neurons. Neurosci Lett 2010; 483: 193–196.
56. Minzenberg MJ, Carter CS. Modafinil: a review of neurochemical actions and effects on cognition. Neuropsychopharmacol Off Publ Am Coll Neuropsychopharmacol 2008; 33: 1477–1502.
57. Ishizuka T, Murakami M, Yamatodani A. Involvement of central histaminergic systems in modafinil-induced but not methylphenidate-induced increases in locomotor activity in rats. Eur J Pharmacol 2008; 578: 209–215.

58. Avelar AJ, Cao J, Newman AH, et al. Atypical dopamine transporter inhibitors R-modafinil and JHW 007 differentially affect D2 autoreceptor neurotransmission and the firing rate of midbrain dopamine neurons. Neuropharmacology 2017; 123: 410–419.
59. Richelson E, Nelson A. Antagonism by antidepressants of neurotransmitter receptors of normal human brain in vitro. J Pharmacol Exp Ther 1984; 230: 94–102.
60. Lim HD. Evaluation of Histamine H1-, H2-, and H3-Receptor Ligands at the Human Histamine H4 Receptor: Identification of 4-Methylhistamine as the First Potent and Selective H4 Receptor Agonist. J Pharmacol Exp Ther 2005; 314: 1310–1321.
61. Gillman PK. Tricyclic antidepressant pharmacology and therapeutic drug interactions updated. Br J Pharmacol 2007; 151: 737–748.
62. Nowak JZ, Arrang JM, Schwartz JC, et al. Interaction between mianserin, an antidepressant drug, and central H1- and H2-histamine-receptors: In vitro and in vivo studies and radioreceptor assay. Neuropharmacology 1983; 22: 259–266.
63. Hatzopoulos AK. Disease Models & Mechanisms in the Age of Big Data. Dis Model Mech 2019; 12(8): dmm041699
64. Jian-Sheng Lin MJ Bernard Roussel, Alexandre Gaspar, Yan Zhao, Yiping Hou, Markus Schmidt, Anne Jouvet. The unfinished journey with modafinil and discovery of a novel population of modafinil-immunoreactive neurons. Sleep Med 2018; 49: 40–52.
65. Gerrard P, Malcolm R. Mechanisms of modafinil: A review of current research. Neuropsychiatr Dis Treat 2007; 3: 349–364.
66. Zager A. Modulating the immune response with the wake-promoting drug modafinil: A potential therapeutic approach for inflammatory disorders. Brain Behav Immun 2020; 88: 878–886.
67. Hashemian SM, Farhadi T. A review on modafinil: the characteristics, function, and use in critical care. J Drug Assess 2020; 9: 82–86.
68. Urban AE, Cubała WJ. The role of eugeroics in the treatment of affective disorders. Psychiatr Pol 2020; 54: 21–33.
69. Brola W, Ziomek M, Czernicki J. [Fatigue syndrome in chronic neurological disorders]. Neurol Neurochir Pol 2007; 41: 340–349.
70. Schoser B. Myotonic Dystrophy Type 2. In: Adam MP, Ardinger HH, Pagon RA, et al. (eds) GeneReviews®. Seattle (WA): University of Washington, Seattle, http://www.ncbi.nlm.nih.gov/books/NBK1466/ (2006, accessed 15 September 2020).
71. Gibbons C, Pagnini F, Friede T, et al. Treatment of fatigue in amyotrophic lateral sclerosis/motor neuron disease. Cochrane Database Syst Rev 2018; 1: CD011005.
72. Yu X, Ma Y, Harding EC, et al. Genetic lesioning of histamine neurons increases sleep-wake fragmentation and reveals their contribution to modafinil-induced wakefulness. Sleep 2019; 42(5):zsz031.
73. Mereu M, Bonci A, Newman AH, et al. The neurobiology of modafinil as an enhancer of cognitive performance and a potential treatment for substance use disorders. Psychopharmacology (Berl) 2013; 229: 415–434.
74. Urbano FJ. Leznik RRLE . Modafinil enhances thalamocortical activity by increasing neuronal electrotonic coupling. Proc Natl Acad Sci 2007; 104: 12554–12559.
75. Miriam Punzi SLS Tommaso Gili, Laura Petrosini, Carlo Caltagirone, Gianfranco Spalletta. Modafinil-Induced Changes in Functional Connectivity in the Cortex and Cerebellum of Healthy Elderly Subjects. Front Aging Neurosci 2017; 30(9): 85.
76. Yates CC, Charlesworth A, Reese NB, et al. Modafinil normalized hyperreflexia after spinal transection in adult rats. Spinal Cord 2009; 47: 481–485.
77. Gellersen HM, Guo CC, O'Callaghan C, et al. Cerebellar atrophy in neurodegeneration-a meta-analysis. J Neurol Neurosurg Psychiatry 2017; 88: 780–788.
78. Sicong Tu, Ricarda MRT, Menke AL, et al. Regional thalamic MRI as a marker of widespread cortical pathology and progressive frontotemporal involvement in amyotrophic lateral sclerosis. J Neurol Neurosurg Psychiatry 2018; jnnp-2018–318625.
79. Ishizuka T, Murotani T, Yamatodani A. Action of modafinil through histaminergic and orexinergic neurons. Vitam Horm 2012; 89: 259–278.
80. Philipp O. Valko TES Yury V Gavrilov, Mihoko Yamamoto, Hasini Reddy, Johannes Haybaeck, Emmanuel Mignot, Christian R Baumann. Increase of histaminergic tuberomammillary neurons in narcolepsy. Ann Neurol 2013; 74: 794–804.
81. Gurney ME, Pu H, Chiu AY, et al. Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. Science 1994; 264: 1772–1775.
82. Apolloni S, Amadio S, Fabbrizio P, et al. Histaminergic transmission slows progression of amyotrophic lateral sclerosis. J Cachexia Sarcopenia Muscle 2019; 10(4): 872–893.
83. Hu W, Chen Z. The roles of histamine and its receptor ligands in central nervous system disorders: An update. Pharmacology and Therapeutics 2017;175:116–132.
84. Volonté C, Morello G, Spampinato AG, et al. Omics-based exploration and functional validation of neurotrophic factors and histamine as therapeutic targets in ALS. Ageing Res Rev 2020; 62: 101121.
85. Volonté C, Apolloni S, Sabatelli M. Histamine beyond its effects on allergy: Potential therapeutic benefits for the treatment of Amyotrophic Lateral Sclerosis (ALS). Pharmacol Ther 2019; 202: 120–131.
86. Mora JS, Genge A, Chio A, et al. Masitinib as an add-on therapy to riluzole in patients with amyotrophic lateral sclerosis: a randomized clinical trial. Amyotroph Lateral Scler Front Degener 2020; 21: 5–14.
87. Kim HY, Moon C, Kim KS, et al. Recombinant human erythropoietin in amyotrophic lateral sclerosis: a pilot study of safety and feasibility. J Clin Neurol Seoul Korea 2014; 10: 342–347.
88. Bedlack R. ALSUntangled 48: Perampanel (Fycompa). Amyotroph Lateral Scler Front Degener 2019; 20: 453–456.
89. Apolloni S, Fabbrizio P, Amadio S, et al. Actions of the antihistaminergic clemastine on presymptomatic SOD1-G93A mice ameliorate ALS disease progression. J Neuroinflammation 2016; 13: 191.
90. Apolloni S, Fabbrizio P, Parisi C, et al. Clemastine Confers Neuroprotection and Induces an Anti-Inflammatory Phenotype in SOD1G93A Mouse Model of Amyotrophic Lateral Sclerosis. Mol Neurobiol 2016; 53: 518–531.
91. Michael J Minzenberg CSC. Modafinil: A Review of Neurochemical Actions and Effects on Cognition. Neuropsychopharmacology 2007; 33: 1477–1502.