Computational and Structural Biotechnology Journal 20 (2022) 1427–1438

COMPUTATIONAL AND STRUCTURAL BIOTECHNOLOGY JOURNAL

journal homepage: www.elsevier.com/locate/csbj

Network-based stage-specific drug repurposing for Alzheimer's disease

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ARTICLE INFO

Article history:
Received 17 November 2021
Accepted 14 March 2022
Available online 16 March 2022

Keywords:
Network-based score
Drug repurposing
Alzheimer's disease

ABSTRACT

Alzheimer's disease (AD) is a progressive neurodegenerative disease and the most common type of dementia. With no disease-curing drugs available and an ever-growing AD-related healthcare burden, novel approaches for identifying therapies are needed. In this work, we propose stage-specific candidate repurposed drugs against AD by using a novel network-based method for drug repurposing against different stages of AD severity. For each AD stage, this approach a) ranks the candidate repurposed drugs based on a novel network-based score emerging from the weighted sum of connections in a network resembling the structural similarity with failed, approved or currently ongoing drugs b) re-ranks the candidate drugs based on functional, structural and a priori information according to a recently developed method by our group and c) checks and re-ranks for permeability through the Blood Brain Barrier (BBB). Overall, we propose for further experimental validation 10 candidate repurposed drugs for each AD stage comprising a set of 26 elite candidate repurposed drugs due to overlaps between the three AD stages. We applied our methodology in a retrospective way on the known clinical trial drugs till 2016 and we show that we were able to highly rank a drug that did enter clinical trials in the following year. We expect that our proposed network-based drug-repurposing methodology will serve as a paradigm for application for ranking candidate repurposed drugs in other brain diseases beyond AD.

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease and is the most common type of dementia. Neurodegenerative diseases are characterized by progressive loss of neurons and function in the central nervous system (CNS). This group of diseases is prevalent to a large proportion of the total population and this prevalence is mainly caused by aging [1]. Due to the increase in life expectancy, the appearance of neurodegenerative diseases, including AD, is predicted to increase worldwide [2]. In 2016, it was reported that AD is affecting ~47 million people, with predictions suggesting to reach ~75 million in 2030 [3]. Moreover, neurodegenerative diseases such as AD carry a great economic burden for public health. For instance, according to Alzheimer's Association in 2017, the money spent for these patients reached $259 billion. Therefore, there is an urgent need to discover and propose novel drugs [4].

Over the past decade, a great effort has been given to understand the pathogenesis and mechanisms involved in neurodegenerative diseases, as well as to discover novel treatments for these diseases. Although a considerably large amount of money has been invested in drug development for AD, only five symptomatic treatments have been approved so far i.e. donepezil (Aricept), galantamine (Razadyne), memantine (Nameda), rivastigmine (Exelon), memantine and donepezil (Namzaric) [5]. The currently approved drugs for AD provide only temporary and incomplete symptomatic relief together with several side effects. Very recently, FDA has conditionally approved the drug Aduhelm (aducanumab), a monoclonal antibody aiming to target the underlying pathophysiology of AD, the presence of β-amyloid peptide (Aβ) plaques in the brain. This therapy aims to slow the progression of the disease, rather than addressing symptoms, and is the first approved therapy of this type. However, aducanumab targeting the plaques, means other aspects of the disease such as neuroinflammation, or the loss of neurons, are still unaffected. Re-examination of clinical trials using high doses of aducanumab showed that the drug might reduce cognitive decline. This re-examination ultimately led to an FDA approval, with the condition that further studies will be conducted to confirm these findings [6,7]. Moreover, in June, the FDA granted lecanemab (BAN2401) a "breakthrough therapy status", a designation that helps accelerate the development of

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https://doi.org/10.1016/j.csbj.2022.03.013

medications for serious and life-threatening conditions. This agreement regarding lecanemab was based on clinical evidence from a Phase 2 (NCT01767311) trial that enrolled 856 patients who had mild cognitive impairment or dementia [8]. The use of the highest dose of lecanemab (for a period of 18 months) showed that brain amyloid was reduced.

Although a high number of AD-related drugs have entered the preclinical phase, the failure rate has been very high due to the complexity of the disease [9]. Various AD-related drugs have failed in clinical trials yet, currently, there are still 486 drugs or combinations of drugs in clinical trials under the recruiting phase (clinicaltrials.gov).

Thorough reviews have been recently carried out by Cummings et al, regarding the record of drugs in clinical trials for the years 2016 to 2020 [10-14]. In this series of work, they used clinicaltrials.gov to determine the number and characteristics of trials in phase I, phase II, and phase III for treatment of AD during the examined period. Although several drugs have gone through clinical trials over the years, only aducanumab was proven to be a promising candidate for reducing the rate of progression of AD, which was recently approved conditionally by FDA.

In order to detect novel and effective treatments for such a complex disease, new methods and approaches are urgently needed that reduce both cost and time. A modern approach in this direction is known as drug repurposing or drug repositioning [15] i.e. the detection of novel indications for existing drugs in order to treat new diseases [16]. Network-based approaches are very useful to drug repurposing since they provide the means of connecting huge amount of data and dealing with its complexity. With respect to drug repurposing, network-based approaches can be used to uncover pairwise relations between drugs, diseases or target proteins. These relations can be depicted by the edges, whereas the various objects are shown schematically as nodes.

In general, there are many studies that have used network-based analysis for drug repurposing. However, for AD, there are limited studies that used this approach so far. Peng *et al* collected the genes associated with the disease and screened potential drugs by a network analysis on the AD-related genes and drug targets in order to search novel candidate drugs for AD [17]. In another study by Zeng *et al*, the authors developed a network-based deep-learning approach, known as deepDR, for *in silico* drug repurposing. Their approach was based on the integration of 10 networks: one drug–disease, one drug-side-effect, one drug–target and seven drug–drug networks. This tool can learn drug features extracted from the aforementioned networks by a multi-modal deep autoencoder. Using this approach, the tool can infer candidate repurposed drugs [18]. Nowadays, accumulation of large volumes of omics data makes drug repurposing an appealing approach for highlighting promising candidate elite drugs for each AD stage. There are several studies available on drug repurposing using transcriptomic data [19-21]. Such an example is a recent study by [21], in which a short list of potential anti-AD drugs was proposed based on computational drug repurposing using gene signatures.

Motivated by the lack of available treatment for AD through traditional drug development approaches, we focused on applying the promising avenue of computational drug repurposing to highlight promising drug candidates. Since each AD stage (defined as incipient: Braak stages I-II, moderate: Braak stages III-IV, severe: Braak stages V-VI) is characterized by different symptoms and molecular mechanisms, we stratified the patient samples based on severity in order to pinpoint stage-specific candidate repurposed drugs.

Here, we present a novel network-based approach, which filters and re-ranks drugs based on different scores, and we propose candidate repurposed drugs for AD. Initially, we performed drug repurposing ananysis for different AD stages (incipient, moderate and severe) using publicly available transcriptomic data. We show that our approach ranks the candidate repurposed drugs based on the sum of their connections of structural similarity with failed, approved or currently ongoing drugs. Moreover, we show the mechanisms of action of our elite candidate repurposed drugs and further comparison with existing knowledge on AD took place. We tested our methodology in a retrospective way on the known drugs till 2016 and we show that we were able to highly rank a drug that did entered clinical trials in the following year.

2. Materials and methods

2.1. Step 1: signature generation

2.1.1. Data

Three AD-related microarray studies from post-mortem brain tissue of human subjects were retrieved from Gene Expression Omnibus (GEO) [22]- a transcriptional data repository. The selection of the datasets was based on the staging of the disease using the Braak indexing, which is used to classify the degree of pathology in Parkinson's disease and AD (Table 1). To our concern, these were the only datasets that clearly used Braak staging of the disease (Braak stage I-II, III-IV, V-VI).

2.1.2. Pre-processing of data

Each dataset selected was quantile normalised and log2 transformed. Subsequent analysis was done in R statistical environment (http://www.R-project.org/) [26]. Each of the three datasets was processed using the Limma R package [27], a linear model that calculates a moderated t-statistic from gene expression experiments.

2.1.3. Detection of differentially expressed genes

After the dataset pre-processing, probe-set IDs were matched to gene symbols according to each platforms' annotation files. We maintained the most differentially expressed ones in cases of gene symbol correspondence to multiple probe-sets. From the Limma analysis result, we kept the top 150 over-expressed and 150 under-expressed genes based on log2FC from the gene list with an adjusted p-value of <0.05. This number of genes (300) corresponds to the input number limit of the drug-repurposing tools we used in the sequel.

2.2. Step 2: Drug re-ranking and short listing

2.2.1. Transcriptomics-based drug repurposing

The transcriptomic-based drug repurposing was performed using four different drug repurposing tools: Connectivity Map (CMap) [28], L1000CDS2 [29], L1000FWD [30] and CRowd Extracted Expression of Differential Signatures (CREEDS) [31]. The 150 over and under expressed genes (based on their FC value) from the three different datasets were used as transcriptomic signatures. Next, each set was used as an input to the aforementioned repurposing tools. The first three tools (CMap, L1000CDS2 and L1000FWD) use transcriptional expression data from multiple human cell lines, to probe relationships between diseases and therapeutic agents. Drugs are sorted according to a score (inhibition score), which characterizes if a drug can reverse (drugs with a strong negative score value) or mimic (drugs with a strong positive score value) the expression levels of a disease based on a given set of genes. For each stage and each dataset, we obtained a candidate list of repurposed drugs predicted by each of the three tools, ranked based on their inhibition score. The fourth of the tools used, CREEDS, contains a list of 4295 single drug perturbations and 8620 single gene perturbations obtained from gene expression data of rat tissues. The same input of genes was used again, yet this time drugs are ranked by the tool based on Fisher's exact test derived

p-value. Finally, from each of the four tools, the top 50 drugs were selected and their union of drugs was used for further analysis.

2.2.2. Collection of the running clinical trials, FDA approved and "failed" drugs of AD

All listed clinical studies related to AD were collected from Cummings et al (2020). Specifically, only small-molecule drugs were obtained from the studies since their 2D -structures were then used for the chemical similarity analysis. Moreover, all listed drugs related to AD that were either withdrawn, suspended, unknown, terminated or completed with no evidence that a certain drug is effective, were collected from the ClinicalTrials.gov. These drugs are referred to as "failed". The Food and Drug Administration (FDA)-approved drugs for AD were also obtained. The SMILES formats of the aforementioned groups of drugs were collected through the PubChem Identifier Exchange Service (https://pubchem.ncbi.nlm.nih.gov/idexchange/idexchange.cgi). We then used the OpenBabel software to convert the SMILES structure format to the 2D-structures of the drugs (sdf files).

2.2.3. CoDRes reranking and scoring

The top 50 drugs generated from each tool and each dataset, were used as input to the CoDReS tool [32]. CoDReS combines an initial inhibition score like the one obtained from the repurposing tools together with a functional score of each drug using the disease of interest and a structural score derived from drugability violations based on the Lipinski [33] and Veber's rules [34]. A composite score (CoDReS score) was calculated for each drug as the weighted normalized sum of the initial inhibition score (aS) with a functional (FS) and a structural score (StS). The weights that determine the desired influence of each part to the final score were defined as waS = 0.45, wFS = 0.45 and wStS = 0.1. The top 50 drugs were then reranked based on the CoDReS score.

2.2.4. Network-based drug repurposing approach

We calculated the structural similarity of the top 50 drugs from each tool per stage against the FDA approved drugs, the failed drugs and the drugs that are currently in clinical trials. The threshold used for the similarity score was > 0.5. Next, we formulated an edge-list based on the similarity score of the drugs. Depending on the type of drug a repurposed drug was connected to, a positive or negative score was multiplied to the weight of their connection, i.e., for connections with (1) failed drugs, the weight was multiplied by −1, (2) with the FDA approved drugs the weight was multiplied by 1 whereas for (3) the drugs that are currently in clinical trials, depending on the phase that the drug is (phase I, II and III), the weight was multiplied by 0.2, 0.4 and 0.6 respectively. Since the pool of failed drugs is bigger than the others (FDA approved and clinical trials), the use of all connections to repurposed drugs would have been biased. Hence, as a final step, only a single connection from each group of drugs (FDA, clinical trials and failed drug) was kept, which was the most highly scored connection (maximum) to each repurposed drug. Once each repurposed drug ended up with a total of five connections (maximum), the strength (i.e., the weighted degree) of each drug node of this network was calculated (Fig. 1).

Moreover, two more parameters are taken into consideration to our overall novel drug repurposing score: the reranking of the repurposed drugs using the CoDReS score and the permeability of the repurposed drugs using the lightBBB tool. LightBBB is a BBB permeability prediction model based on Light Gradient Boosting Machine (LightGBM) algorithm [35]. Permeability score given is either permeable or non-permeable. The overall scoring is represented from the equation:

$$\boldsymbol{DRs} = \boldsymbol{wCT} * \boldsymbol{vCT} + \boldsymbol{wBBB} * \boldsymbol{vBBB} + \boldsymbol{wCoDReS} * \boldsymbol{vCoDReS} \quad (1)$$

where **vCT** is the normalized score of the repurposed drugs based on their similarity with failed, FDA and clinical trials, **vBBB** is the prediction whether a drug is permeable or not and **vCoDReS** as obtained from the CoDReS tool, and **wCT, wBBB** and **wCoDReS** are the weights given to each score, 0.6, 0.1 and 0.3 respectively.

2.3. Step 3: validation analysis

2.3.1. Pathway analysis

Drug targets of the elite repurposed drugs per stage were collected through Drug Central (https://drugcentral.org) and NCATS Inxight: Drugs (https://drugs.ncats.io/). For drug targets, a cut-off rule using the empirical value of K_D > 6 (-logM);1μM was used. Moreover, the curated genes associated with AD were collected through DisGeNET, a platform containing one of the largest publicly available collections of genes associated to human diseases [36]. A threshold of gda score>=0.5 was used in order to keep the most important genes associated to AD. All genes (drug targets and genes associated to AD) were used as an input to our in-house tool PathIN (https://bioinformatics.cing.ac.cy/PathIN/). PathIN holds a large database repository of reference pathway networks, across a large set of species, which have been developed through the freely available information included in the KEGG, Reactome, and Wiki Pathways database repositories. It is used for finding subnetworks of pathways that are related to a given set of genes.

2.3.2. Structural similarity among the 26 elite repurposed drugs

ChemBioServer 2.0 (https://chembioserver.vi-seem.eu/) is a publicly available web application that provides filtering, clustering, comparing drug structures and networking of chemical compounds facilitating both drug discovery and repurposing [37,38]. Hierarchical clustering using Tanimoto similarity (Soergel distance $\leq$ 0.4) with a clustering threshold set to 0.4 was applied to the final list of all drugs gathered.

3. Results

We developed a novel network-based drug repurposing approach, as illustrated in Fig. 1. The overall process entails the analysis of stage-specific AD related microarray datasets to identify significant genes, with the subsequent identification and shortlisting of candidate repurposed drugs and the pathways they target (see Fig. 1). This approach computes a novel clinical testing (CT) score (clinical testing) that is calculated through the structural similarity of the candidate repurposed drugs to either previously failed drugs, or currently FDA approved or currently tested drugs in clinical trials (see Methods for more details).

3.1. Drug repurposing

The first part of this study included the collection and analysis of publicly available microarray datasets of AD patients and controls. Following the preprocessing of these datasets we performed differential analysis to identify differentially expressed genes (DEG) between the two conditions. We used a cut-off of p adj. < 0.05 and then sorted the differentially expressed genes based on their log2 fold-change (log2FC) value. We selected the top 150 over- and 150 under-expressed genes as it is a requirement for most of the repurposing tools.

Using the DEG sets, we performed a series of *in silico* drug repurposing analyses with existing computational tools (see Methods): Connectivity Map (CMap) [28], L1000CDS2 [29], L1000FWD [30] and CRowd Extracted Expression of Differential Signatures (CREEDS) [31], leading to four lists of candidate repurposed drugs

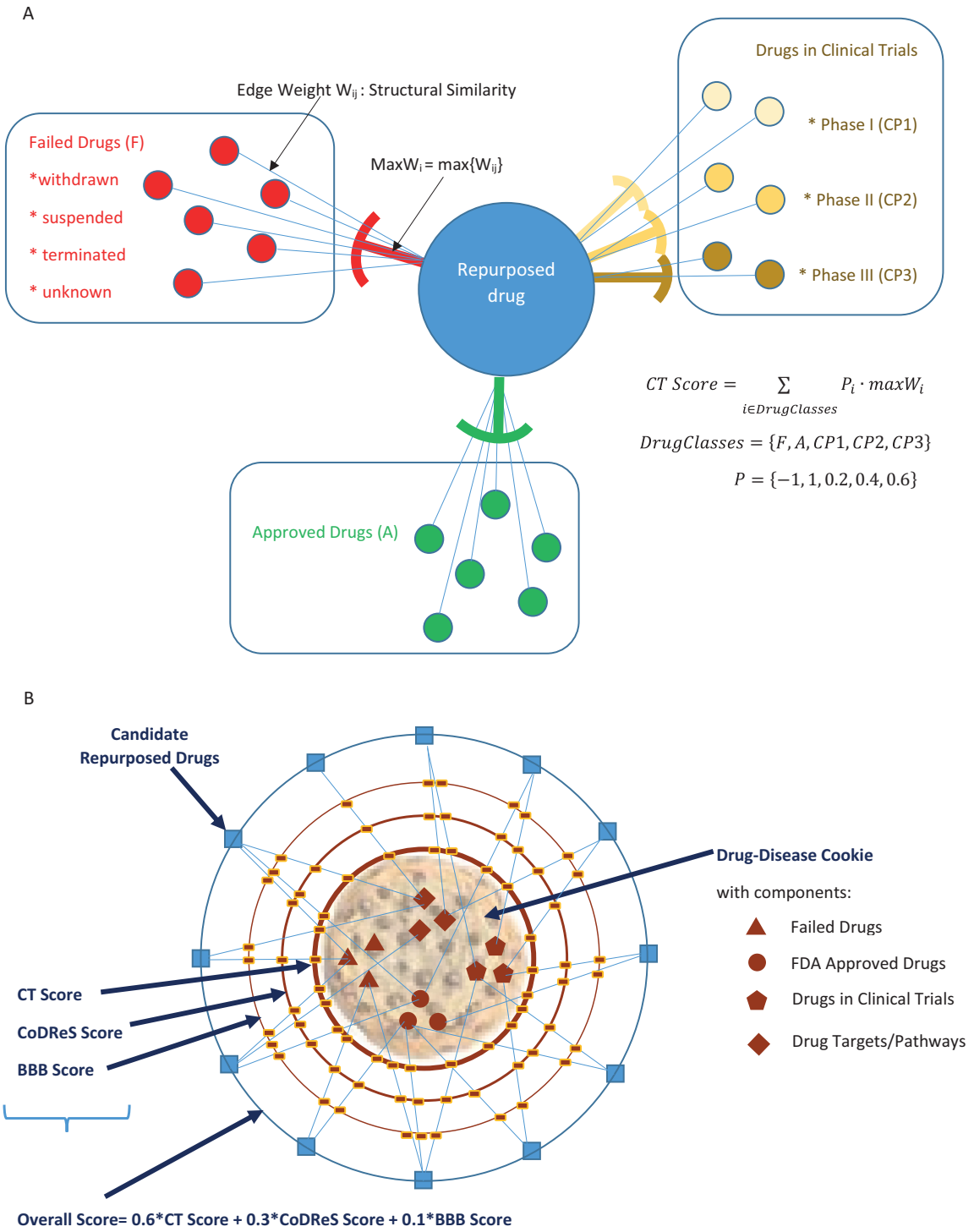

Fig. 1. A. Network-based drug repurposing approach. A positive weight 1 was assigned between candidate repurposed and approved drugs, a negative weight −1 between candidate repurposed and failed drugs, a 0.2 score between candidate repurposed to phase I clinical trials, a 0.4 score between candidate repurposed to phase II clinical trials and a 0.6 score between candidate repurposed to phase III clinical trials. **B.** Disease cookie showing in the center the components used for the creation of the network-based approach (failed, approved drugs, drugs in clinical trials and drug targets/pathways), and around it the CT score, permeability through the BBB and the use of CoDReS giving an overall score, and their connection to the candidate repurposed drugs.

(we kept the top 50 from each list), for each stage of AD severity (defined as incipient: Braak stages I-II, moderate: Braak stages III-IV, severe: Braak stages V-VI). This process was performed for three different AD microarray datasets.

Following the pipeline illustrated in Fig. 2, we selected the top 10 drugs per stage (incipient, moderate and severe) ending up with a group of 26 candidate repurposed drugs for all stages. Interestingly, some drugs were found to be fitting for two stages, such as *emetine* and *omacetaxine mepesuccinate* both for incipient and moderate stages, and *piperidolate* and *paroxetine* for moderate and severe stages (see Table 1 for the full list of top 10 drugs per AD stage).

3.2. Short-listing of candidate repurposed drugs using a novel network-based approach

Following the identification of the candidate repurposed drugs per stage we performed a ranking, based on the combination of three complementary methods. Initially, in our drug repurposing pipeline we exploited the network connectivity of the drugs in a structural similarity network using the Tanimoto similarity. To detect potential AD drugs through this approach, we used an in-house script using the Rcpi package in R to calculate the structural similarity between the initial list of candidate repurposed drugs with the structures of the currently FDA approved, previously failed (withdrawn, suspended, terminated, unknown or completed) and drugs currently in clinical trials for the year 2020 for AD and we initially kept all connections with structural similarity above 50%. We devised a novel network-based method which identifies drugs that are more similar to approved drugs or drugs that are currently in clinical trials, and less similar to previously failed drugs. To do so, we assigned a positive weight of score 1 between candidate repurposed and approved drugs, a negative weight of score −1 between candidate repurposed and previously failed drugs and a positive score of a lower impact between candidate repurposed and drugs in clinical trials (phase I score of 0.2, phase II score of 0.4 and phase III score of 0.6). These scores were then multiplied with the existing weight of the connections of the drugs (more details see Methods section).

The second approach in our drug repurposing pipeline involved the re-ranking of the candidate drugs using CoDReS tool [32] based on (a) a functional score combining the drug targets' relevance to the disease and the binding affinity to its target genes, (b) an *a priori* score defined as the normalized initial drug ranking from each list, and (c) a structural score representing drugability violations. We performed the CoDReS reranking on the top 50 drugs of each list from each dataset. For each stage (stage I-II, III-IV, V-VI), the average score for drugs appearing in two or more lists was calculated.

The third approach in our drug repurposing pipeline focuses on the calculation of the permeability of the candidate repurposed drugs through the BBB using a prediction tool, named "lightBBB". This was done separately for the candidate repurposed drugs of each of the AD stages. By combining all three parts of the repurposing approach, we ended up with a final scoring scheme that takes into consideration 1) the novel network-based score, 2) the re-ranking of drugs using the tool CoDReS and 3) the permeability of drugs using the lightBBB tool. The re-ranked lists based on the three aforementioned scores are given in Supplementary tables 1-3.

Based on the composite score combining all approaches, the 10 top-scoring drugs were chosen for each AD stage (Table 2, 3 and 4) whereas full lists of drugs are shown in Supplementary (Table 2-4). Hence, we shortlisted the 26 top-scored drugs from the union of

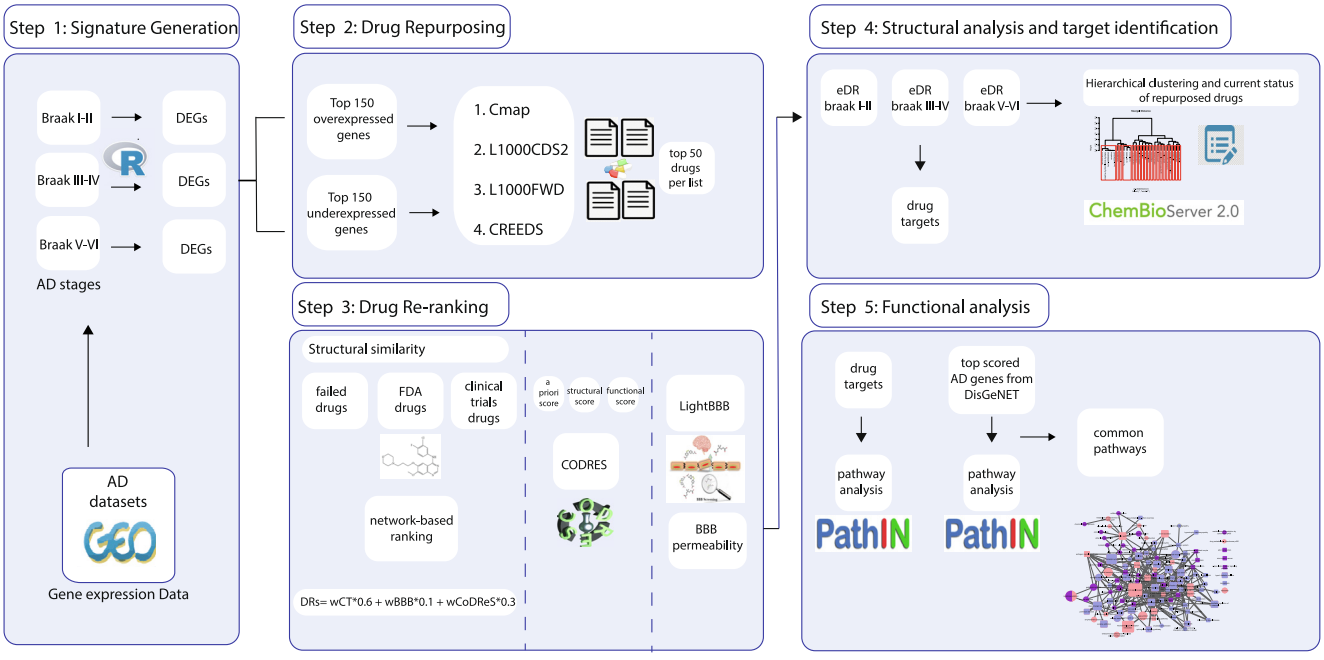

Fig. 2. Analysis Pipeline. Step1: Signature Generation: Initially, each GSE dataset is processed with Limma R package to find differentially expressed genes (DEGs). This is done for each stage of AD separately; Braak stage I-II (incipient), Braak stage III-IV (moderate), Braak stage V-VI (severe). Step 2: Drug repurposing: The generated lists of over- (top 150) and under-expressed genes (top 150) are used as input in the four drug repurposing tools. Top 50 drugs were selected from each tool from each dataset. Step 3: Drug Re-ranking: A three-branch approach is then followed: the network-based approach, which ranks drugs based on a normalized CT score (see Methods), the CoDRes (Composite Drug Reranking Scoring) re-ranking of candidate repurposed drugs and the drug permeability through the BBB using the tool LightBBB. Top 10 drugs per stage (incipient, moderate and severe) were selected, comprising a group of 26 drugs. Step 4: Structural clustering and target identification: hierarchical clustering of the 26 candidate repurposed drugs was performed. Moreover, the current status of candidate repurposed drugs in clinical trials was recorder. Finally, drug targets of our elite candidate repurposed drugs were detected. Step 5: Functional analysis: Pathway analysis of drug targets of our elite candidate repurposed drugs as well as pathway analysis of top-curated genes of AD from DisGeNET was performed to detect pathways that are strongly connected to the disease.

Table 1
The experimental design of the microarray data sets that were used in this study.

No	Author	GEO accession number	Control Number	Patient Number	Pathologic disease stage
1	[23]	GSE36980	47	32	Braak V-VI
2	[24]	GSE110226	6	7	Braak V-VI
3	[25]	GSE483650	173	80	Braak I-II Braak III-IV Braak V-VI

Table 2
Top 10 candidate repurposed drugs of stage I and II for AD. The Combined score corresponds to our calculated score combining the CT score, the CoDReS score and the BBB permeability score. Drug targets were extracted from DrugCentral (https://drugcentral.org/) and NCATS Inxight: Drugs (https://drugs.ncats.io/).

Candidate repurposed drug	Combined score	Drug targets
Clomifene	0.873	*ESR1, ADRA2A, SLC6A4, SLC6A2, HTR2A, HTR2B, HTR2C, ADRA2B, DRD3, CHRM1, CHRM2, CHRM3, CHRM4, CHRM5, ADRA2C, SLC6A3, EGFR, TACR2, SIGMAR1, HTR6, EBP*
Camptothecin	0.823	*TOP1*
Omacetaxine mepesuccinate	0.790	*RPL3, RPL2*
Emetine	0.778	*ADRA2A, ADRA2C, ADRA1D, ADRA1B*
Pik-90	0.759	*PI3K*
Oxibendazole	0.747	*TUBB, TUBA1A*
Cephaeline (brd-k80348542)	0.741	*HTR4*
Vinpocetine	0.739	*PDE1A, PDE1C*
Emetine hydrochloride hydrate	0.719	*ADRA2A, ADRA2C, ADRA1D, ADRA1B*
Vapiprost	0.718	*TBXA2R*

Table 3
Top 10 candidate repurposed drugs of stage III and IV for AD. The Combined score corresponds to our calculated score combining the CT score, the CoDReS score and the BBB permeability score. Drug targets were extracted from DrugCentral (https://drugcentral.org/) and NCATS Inxight: Drugs (https://drugs.ncats.io/).

Candidate repurposed drug	Combined score	Drug targets
Paroxetine	0.790	*SLC6A4, SLC6A2, SLC6A3, CHRM1, CHRM2, CHRM3, CHRM4, CHRM5, TACR1, SIGMAR1, CYP2D6*
Gatifloxacin	0.787	*GYRA-STRPN, GYRB-STRPN, PARC-STRPN, PARE-STRPN*
Piperidolate	0.783	
Omacetaxine mepesuccinate	0.769	*RPL3, RPL2*
Ro 04–5595	0.764	*GRIN1*
Naloxone hydrochloride	0.762	*OPRM1, ADORA3, OPRD1, OPRK1, TLR4, SIGMAR1, CCR5*
Ethosuximide	0.761	*CACNA1G*
Perindopril	0.760	*ACE*
Brd-k80346834	0.743	
Emetine	0.739	*ADRA2A, ADRA2C, ADRA1D, ADRA1B*

Table 4
Top 10 candidate repurposed drugs of stage V and VI for AD. The Combined score corresponds to our calculated score combining the CT score, the CoDReS score and the BBB permeability score. Drug targets were extracted from DrugCentral (https://drugcentral.org/) and NCATS Inxight: Drugs (https://drugs.ncats.io/).

Candidate repurposed drug	Combined score	Drug targets
Tetrabenazine	0.895	*SLC18A2, ADRA2A, ADRA2C*
Iloperidone	0.892	*HTR2A, DRD2, DRD3, HRH1, HTR1D, DRD4, HTR6, HTR7, ADRA1A, HTR2C, SIGMAR1, HTR1A, HTR1B, ADRA2A, ADRA2B, ADRA2C, SLC6A2*
Hydrocotarnine	0.828	*CYP2D6, CYP1A2*
Bi-2536	0.821	*PLK1*
Scoulerine	0.814	*ADRA1D, ADRA2A, GABRA1*
Piperidolate	0.810	*CHRM1*
Mepacrine	0.794	*CHRM3, CHRM4, CHRM2, CHRM1, DRD3, ADA2C, HTR2A, ADRA2B, ADRA2A, ADRA1D, PRNP*
Noscapine	0.781	
Paroxetine	0.779	*SLC6A4, SLC6A2, SLC6A3, CHRM1, CHRM2, CHRM3, CHRM4, CHRM5, TACR1, SIGMAR1, CYP2D6*
Lomerizine	0.773	*CACNA1B*

the three main lists. Using a score of 0.6 for the normalized CT score, 0.3 for the CoDReS score and a 0.1 for the permeability through the BBB score, we identified *clomifene, camptothecin, omacetaxine mepesuccinate* as the top scoring drugs for incipient AD (stage I-II), *paroxetine, gatifloxacin* and *piperidolate* as the top scoring drugs for moderate AD (stage III-IV) and *tetrabenazine, iloperidone* and *hydrocotarnine* as the top scoring drugs for severe AD (stage V-VI) from the network-based approach. However, *hydrocotarnine*, a crystalline alkaloid is not a medicinal drug since its actions on CYP enzymes are not being used therapeutically in the clinic. Moreover, *emetine hydrochloride hydrate*, a drug detected in Braak stage I-II involves the same active drug as *emetine*, only in a different solubility form, hence it should not make a difference in terms of targeting. We performed a one-sided Fisher's exact test by

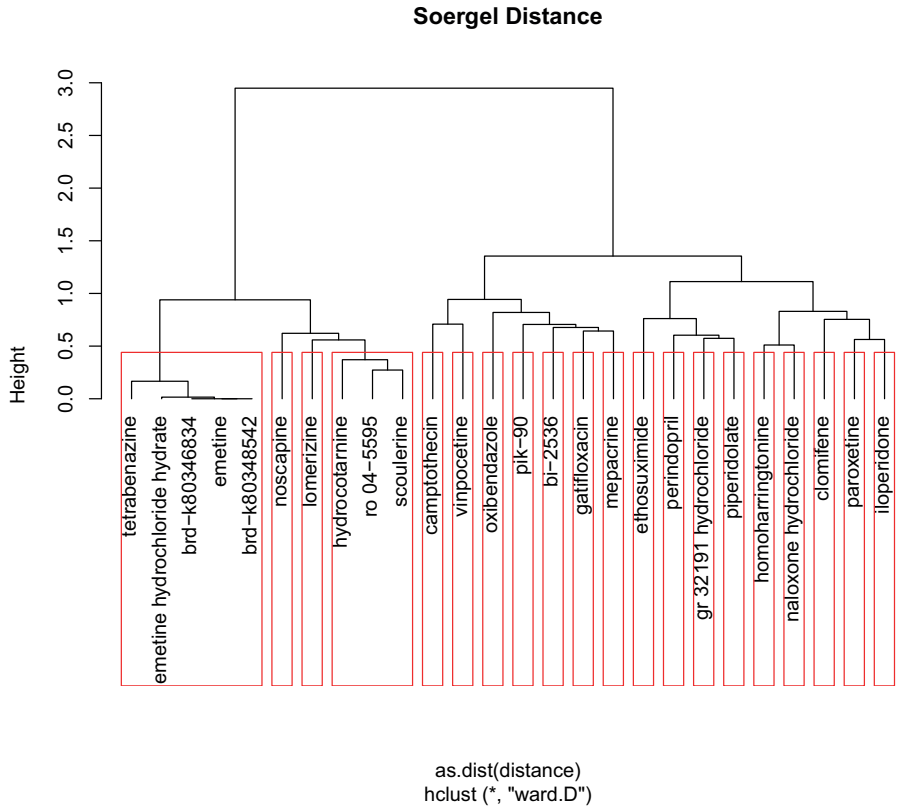

Fig. 3. ChemBioserver hierarchical clustering of the final candidate elite repurposed drugs (26) based on structural similarity. The clustering threshold was set to 0.4. The total of 26 drugs are placed into 20 different drug groups.

comparing the whole library of drugs and the 26 candidate repurposed drugs and these results showed a $p < 0.05$, suggesting that the detection of these 26 candidate repurposed drugs was significant and not randomly selected. Hence, we are proposing that the drugs detected from the combination of the three approaches described should be investigated further for their potential therapeutic efficacy in AD.

3.3. Hierarchical clustering among the candidate elite 26 repurposed drugs for AD

Structural similarity among the candidate elite repurposed drugs of the final lists was calculated using the ChemBioServer [38]. A hierarchical clustering based on Soergel distance and Ward linkage was carried out (see Methods), from which 20 different drug groups were produced (Fig. 3). Here we can see that drugs such as *tetrabenazine, emetine hydrochloride hydrate, brd-k80346834, emetine, brd-k80348542* appear in the same drug group, as well as *hydrocotarnine, ro 04–5595* and *scoulerine*, which also appear in a same drug group. The rest of the 18 drugs are present in different drug groups. These results suggest that the majority of our proposed repurposed drugs are quite distant, i.e. not very similar between them, indicating lack of redundancy in our proposed drug set.

3.4. Current status of top repurposed drugs

We selected the top-5 candidate repurposed drugs from each stage to assess their current status in clinical trials. Of the top-15 candidate repurposed drugs in total, none of them have been

Table 5
Current clinical trial status of top 5 candidate repurposed drugs of each Braak stage.

Candidate repurposed Drug	Clinical trial registration number	AD Stage
Clomifene	NCT04306692	I-II
	NCT04887402	
	NCT02436226	
	NCT04157725	
	NCT03245827	
	NCT02890238	
	NCT02330757	
Camptothecin	N/A	I-II
Omacetaxine mepesuccinate	NCT04505995	I-IV
	NCT04248595	
	NCT04083911	
	NCT04126681	
Emetine	N/A	I-II
Pik-90	N/A	I-II
Paroxetine	NCT04757571	III-IV
	NCT03894085	
	NCT03277339	
	NCT04188028	
	NCT04310579	
	NCT04404439	
	NCT04218981	
Gatifloxacin	NCT03696342	III-IV
Piperidolate	N/A	III-IV
Ro 04-5595	N/A	III-IV
Tetrabenazine	NCT02509793	V-VI
Iloperidone	NCT04819776	V-VI
	NCT04712734	
Hydrocotarnine	N/A	V-VI
Bi-2536	N/A	V-VI
Scoulerine	N/A	V-VI

tested in clinical trials for AD yet. However, drugs such as *camptothecin, vinpocetine, paroxetine, naloxone* and *mepacrine* have been tested in pre-clinical AD models [39-42]. Table 5 shows a summary of all the clinical trials that each candidate repurposed drug is involved. Seven out of the 14 drugs were not involved in clinical trials at all (as reported by clinicaltrials.gov).

For instance, *clomiphene* is an elective estrogen receptor modulator and is used as a treatment for subfertility due to anovulation while *camptothecin* is used in cancer therapy. *Omacetaxine mepesuccinate* is a unique agent approved by the FDA for the treatment of chronic myeloid leukemia. Paroxetine is an antidepressant whereas *gatifloxacin* is a fourth-generation fluroquinolone antibiotic used as a topical application for ophthalmic conditions. Moreover, *emetine*, a natural alkaloid, has been used in phytomedicine to induce vomiting and treats cough and severe amoebiasis. In addition, *iloperidone* is an atypical antipsychotic used in schizophrenia while *scoulerine* is an isoquinoline alkaloid, with promising suppressive effects of cancer cell growth *in vitro*. Notably, *tetrabenazine* is the only FDA drug for the treatment of chorea related to Huntington's disease and other hyperkinetic disorders. Huntington's disease is another neurodegenerative disease, with some common aspects and molecular mechanisms to AD.

3.5. Computational insights on the validity of the findings

Following the identification of the short-listed candidate repurposed drugs for each AD-stage we compared our findings with *a priori* AD-related knowledge. This *meta*-analysis was performed in order to validate the novel network-based method using the mechanisms of action of our candidate repurposed drugs with existing, known mechanisms of action involved in AD and AD drug data from previous years.

Firstly, we detected the pathways related to the drug targets of our elite candidate repurposed drugs, using the PathIN tool, an in-house tool (https://bioinformatics.cing.ac.cy/PathIN/), for finding subnetworks of pathways that are related to a given set of genes. Drug targets were in a total of 62 unique genes, leading to possible association of 135 pathways. From these 135 pathways we then detected common pathways with the ones generated using curated AD-related genes, acquired from DisGeNET. For the DisGeNET genes, we used a threshold of gene disease association (gda) score>=0.5, resulting in 38 genes and 159 pathways. This analysis resulted in 95 common pathways between the two pathway lists, 64 unique to DisGeNET list and 40 unique to the drug targets of elite candidate repurposed drugs list. Moreover, pathways that share common genes are also shown in Table 4 of the Supplementary Material. Fig. 4 shows the resulting pathways using the drug targets of our elite candidate repurposed drugs. Square nodes show common pathways between drug targets and DisGeNET gene lists. The rest of the nodes represent pathways that are unique to drug targets. Interestingly, most of the pathways that are unique to drug targets of our elite candidate repurposed drugs, are targeted by drugs detected in stage Braak V-VI (severe) of AD (nodes with purple color) (Fig. 4). 37 pathways are targeted by drugs of Braak stage

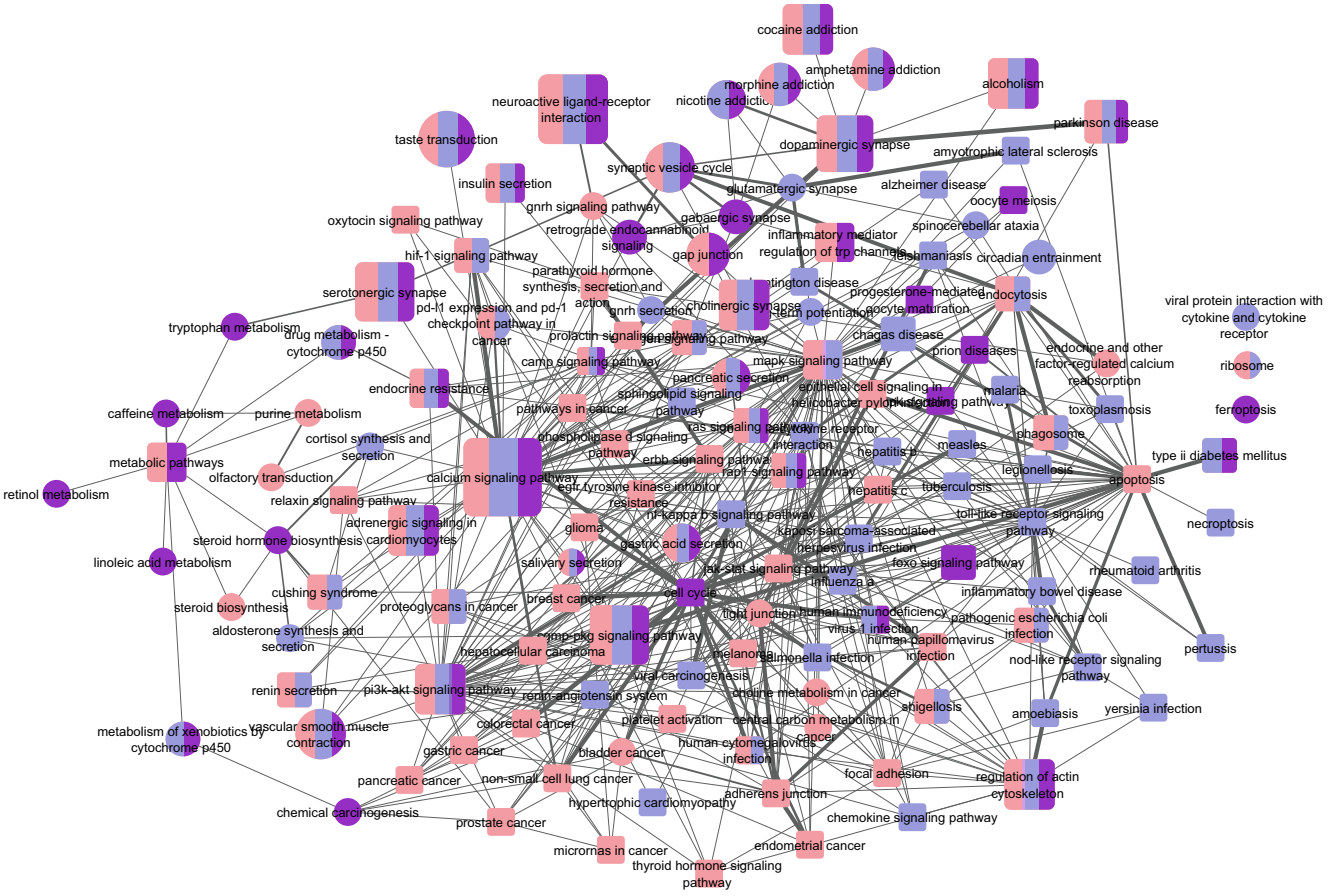

Fig. 4. Pathway-pathway network. Using the drug targets of the candidate repurposed drugs we detected the pathways that these genes are associated with and created a pathway-pathway network. The 135 pathways that are targeted by our elite candidate repurposed drugs share 95 common pathways with the ones from DisGeNET, which give a total of 159. Common pathways are shown in square nodes, whereas unique to drug targets are shown in circles. Different colors show pathways targeted from candidate repurposed drugs from Braak stage I-II, III-IV and V-VI. Pink colored nodes show pathways targeted by drugs from stage I-II, light blue pathways targeted by drugs from stage III-IV and purple pathways targeted by drugs from stage V-VI. The size of nodes corresponds to the number of drugs targeting each pathway. The width of edges corresponds to the number of shared genes between pathways.

Table 6
List of candidate repurposed drugs that are targeting the detected pathways using PathIN. Information regarding their mechanism of action and pathways are included. MOAs were collected through DrugEnrichr (https://maayanlab.cloud/DrugEnrichr/).

Drug name	Mechanism of action	Braak stages	No. of pathways	DisGeNET pathways
Ethosuximide	Voltage-dependent T-type calcium channel subunit alpha-1G inhibitor	III-IV	8	4
Mepacrine	NFkB pathway inhibitor	V-VI	20	13
Lomerizine	Calcium channel blocker	V-VI	11	6
Vinpocetine	Phosphodiesterase inhibitor sodium channel blocker	I-II	8	4
Paroxetine	Selective serotonin reuptake inhibitor (SSRI)	III-VI	20	12
Iloperidone	Serotonin receptor antagonist dopamine receptor antagonist	V-VI	19	14
Scoulerine	Adrenergic receptor antagonist GABA receptor antagonist serotonin receptor antagonist	V-VI	11	4
Noscapine	Tubulin polymerization inhibitor	V-VI	–	–
Piperidolate	Acetylcholine receptor antagonist	III-VI	6	6
Naloxone hydrochloride	Opioid receptor antagonist	III-IV	39	38
Tetrabenazine	Reversible human vesicular monoamine transporter type 2 inhibitor	V-VI	9	7
Bi-2536	PLK inhibitor apoptosis stimulant cell cycle inhibitor protein kinase inhibitor	V-VI	2	2
Hydrocotarnine		V-VI	11	3
Clomifene	Estrogenic agonist/antagonist	I-II	64	50
Perindopril	Angiotensin converting enzyme inhibitor	III-IV	4	4
Oxibendazole	Tubulin polymerization inhibitor	I-II	5	3
Vapiprost	Thromboxane receptor antagonist prostanoid receptor antagonist	I-II	3	3

I-II, 37 by drugs of Braak stage III-IV, whereas only 15 are targeted by drugs of Braak stage V-VI. Notably, 25 pathways are targeted by drugs of all three stages. Of the pathways that were targeted by drugs of all three stages, calcium signaling pathway and pi3k-akt signaling pathway were in the top 3 pathways showing the highest degree (Supplementary table 5).

Key network metrics of the pathway-pathway network were calculated using the Cytoscape plug-in Network Analyzer, to reveal pathway nodes that are important based on the topology of the generated network. These metrics were calculated in order to detect important pathways based on their communication with others, how strongly connected they are etc. All 135 pathways detected, along with the different metrics calculated are listed in supplementary table 5.

To further analyze and interpret the findings from the pathway network, the candidate repurposed drugs that are targeting the detected pathways were used as an input to DrugEnrichr and their mechanisms of action were detected. The disease stage of the candidate repurposed drugs is also mentioned, as well as the number of pathways that each drug is targeting and how many of those are also detected through a-priori knowledge for AD (DisGeNET) (Table 6). From the results in table 6, calcium signaling pathway appears as the pathway with highest degree for six out of the seventeen candidate repurposed drugs, a pathway that appears to be targeted in all three Braak stages of AD.

3.6. Retrospective analysis on the 2016 drugs

A retrospective computational experiment was performed to testify the validity of our findings. We re-applied the network-based approach developed herein, by simulating the drug repurposing status in the year of 2016. We used the information regarding the failed drugs up to the year of 2016 and drugs in AD clinical trials of the year 2016. This validation step was carried out in order to show whether our approach could predict candidate repurposed drugs that entered AD clinical trials in the year of 2016 and upwards. From the top 30 candidate repurposed drugs detected, 6 drugs (*candesartan, saracatinib, nicotine, pioglitazone, vorinostat* and *curcumin)* were detected to appear in clinical trials in the following years (Table 7). Specifically, *candesartan* appeared in AD clinical trials in all four years. Moreover, *pioglitazone*, which was already in phase III AD clinical trials in 2016, was also detected in 2017. However, *pioglitazone* is currently a failed drug for AD. *Curcumin*, a natural product, has been in clinical trials since 2019

Table 7
Course of the drugs detected in 2016 over the years. The Braak stage, which the drug is targeting, is shown, as well as the presence of the drug in the next clinical trials.

Repurposed Drugs of 2016	Braak stage	Clinical trials 2016	Clinical trials 2017	Clinical trials 2018	Clinical trials 2019	Clinical trials 2020
Candesartan	V-VI		yes	yes	yes	yes
Saracatinib	V-VI		yes	yes		
Nicotine	III-IV		yes		yes	yes
Pioglitazone	all	yes	yes			
Vorinostat	V-VI			yes	yes	Yes
Curcumin	V-VI				yes	Yes

and is still ongoing. A remarkable result was the detection of *saracatinib*, which was the highest scoring drug from the ones detected, yet was discontinued in 2018 for AD. However, its prediction using our novel network-based approach showed a very high score, reaching the top 10 drugs of that year (Supplementary table 6–8). *Nicotine* has been discontinued in 2018, however a clinical trial with nicotine transdermal patch was still ongoing in 2020 clinical trials, since 2019.

4. Discussion

Here, we propose a novel network-based drug repurposing approach for AD stages aiming to further filter and prioritize candidate repurposed drugs to be short-listed for further experimental validation in the future. Our analysis of AD gene signatures from transcriptomic datasets included a scoring scheme of three different approaches; the network-based approach resulting in the normalized CT score, the CoDReS score and whether the drugs are permeable or not through the BBB. This approach resulted in a total of 26 elite candidate repurposed drugs against three different severity stages. Computational and literature-based analysis showed 95 common pathways hosting the drug targets of candidate repurposed drugs and top curated AD-related genes from DisGeNET. The highlighted drugs were shown to be generally structurally different among them, meaning that most of the drugs proposed do not belong in the same structural sub-group. Performing a computational experiment as if we were back to 2016, we repeated the same process with the known drug information of 2016 and detected the drug *saracatinib* as a candidate, which then entered clinical trials for AD in 2017. The novelty of this study lies on setting the focus of drug repurposing on AD staging by applying a novel network-based approach that filters and re-ranks repurposed drugs based on structural properties and their similarities to drugs that either failed or drugs that are currently in clinical trials.

In 2014–2019, *saracatinib* was tested in phase-II multi-center trial (NCT02167256) in order to evaluate the safety, tolerability, and its effectiveness in treating patients with a mild AD. The study followed patients for 52 weeks and observed if there were changes in the brain's metabolism of glucose, cognitive and functional measures, and the size of brain areas through imaging techniques. None of the measures showed a statistical difference between the treatment and placebo groups but there was a trend towards less shrinkage in the hippocampus and entorhinal regions of the brain in the saracatinib group.

Moreover, *pioglitazone*, a drug that was already in clinical trials in 2016, was detected as a candidate through our approach using 2016 data. However, *pioglitazone* was terminated for its use for AD in 2018 since according to published trial results, it did not delay the onset of Mild Cognitive Impairment. The failure of this diabetic drug to be used in humans was mostly contributed to the poor BBB permeability as well as to serious peripheral side effects [43]. Although this drug was terminated, reassessment of its use is considered. For instance, Jojo *et al*, performed a study in which they formulated and optimized intranasal nano lipid carriers of *pioglitazone* for its targeted delivery to the brain [43]. The detection of this drug through our approach by using both data from 2016 and 2020, as well as the presence of studies trying to optimize its use for AD, might be a prove that the drug could still be a possible candidate for the disease if several changes and optimization processes take place.

For AD, the number of genetic factors that are key contributors to the disease are several, yet even those cannot fully explain the totality of AD cases. Rather than single genes, a better approach would be investigating AD as a pathology related to alterations affecting entire biological pathways. Detection of pathways such as calcium signaling pathway, cholinergic synapse, dopaminergic synapse, GABAergic synapse, gap junction, are only some of the pathways that were detected from the drug targets of our elite candidate repurposed drugs. These pathways are well-known to be involved in the underlying mechanisms of actions of the disease [44]. Moreover, dysregulation of the MAPK signaling pathway, one of the top detected pathways in our analysis, has been associated with AD [45].

Regarding the drug targets of the candidate repurposed drugs, *ACE, ESR1* and *CYP2D6* were found to be common with the top curated genes for AD detected from DisGeNET. Specifically, *ACE* scored second in the DisGeNET list, showing a strong association with the disease. ACE encodes angiotensin I converting enzyme (ACE1), which regulates blood pressure through the renin-angiotensin system. However, ACE and its mechanistic relationship to AD is complex. It has been shown that by inhibiting ACE1 in the hippocampus of rodents, this enhances their memory [46], decreases memory deficits in mouse models of AD, and also reduces the incidence of AD in humans, as supported by the literature. Changes to ACE1 function by using ACE inhibitors could potentially change AD risk by affecting blood pressure since it is correlated to AD and hence several ACE inhibitors have been in clinical trials for the disease [47]. Moreover, for the *ESR1I* gene, which encodes the estrogen receptor alpha, several SNPs have been described and some of these SNPs have been related to AD in previous studies [48,49] Lastly, CYP2D6 is one of the enzymes involved in drug metabolism. Several studies have shown that polymorphisms in this specific gene can induce alteration in drug metabolism and hence can modify the drug's efficiency and safety. More specifically, this enzyme has been shown to be one of the main CYP enzymes involved in the metabolism of *donepezil*. In particular, polymorphisms of the CYP2D6 seem to play a role in the pharmacokinetics of *donepezil*, which may influence the efficacy of treatment in patients with AD [50].

Regarding the detected candidate repurposed drugs, *erindoprilerbumine (perindopril)* is a prodrug ester of *perindoprilat*, which is an ACE inhibitor [51]. Moreover, according to MesH, *clomiphene* is a triphenyl ethylene stilbene derivative, an estrogen agonist or antagonist. Hence, it targets the gene *ESR1,* a gene correlated with AD as previously mentioned.

Iloperidone, an atypical antipsychotic, has a key mechanism of action the antagonism of certain receptors, such as dopamine D2 and serotonin 5-hydroxytryptamine (5HT) receptor 2A and is being approved to treat schizophrenia. As an antipsychotic, this drug could be a potential candidate for symptom-relief for AD since antipsychotics are often prescribed to patients. It shows promising efficacy and safety profile compared to other drugs [52].

Depression is another symptom that patients with dementia or AD might develop. *Paroxetine*, as detected from our repurposing methodology, is a selective serotonin reuptake inhibitor and according to the literature, it is one of the most common off-label drugs used in daily clinical practice [53]. Hence, it could be a good candidate for treating people with AD that have depression.

In this work, we present an approach that detects small molecules as candidate treatment for AD. However, there is also a group of drugs, monoclonal antibodies that are also candidates in clinical trials for AD. As previously mentioned, FDA has approved as an emergency treatment the antibody *aducanumab*, aiming to target at the underlying pathophysiology of AD, the presence of Aβ plaques in the brain. Although antibodies represent a rather large group of candidate drugs they were not part of the scope of this current work, which focused on small molecules.

A limitation of the present study is that it is based on the gene-signatures derived from post-mortem AD patient specimens. Hence, some of the perturbations detected could be a result of hypoxia and not related to AD. However, this is usually the case

when analyzing brain samples related to neurodegenerative diseases due to the limited number of available data from patients antemortem.

Network-based approaches have been proven to be significant in drug repurposing as it is observed by various studies [54,55]. One such example was presented by [17] in which they propose a network-based systematic computational process to discover new drugs implicated in AD based on AD-related genes, drug targets, and further filtering of the drug candidates regarding their gene expression profiles. Our network methodology as developed here has several strengths since it ranks the candidate repurposed drugs based on their functional association with the disease, their structural diversity, their BBB permeability and their overall similarity with previously failed, approved or currently in clinical trials AD drugs. Thus, the presented methodology provides a comprehensive short list of candidate repurposed drugs with a resolution regarding AD stages that can be further validated experimentally in the future.

5. Conclusions

We performed drug repurposing analysis across different AD stages and we proposed a novel network-based method for ranking candidate repurposed drugs, together with combining the already established method of CoDReS for drug re-ranking and the drug permeability prediction using the tool lightBBB. Through this approach, we derived an elite group of candidate repurposed drugs per AD stage. Finally, using the drug targets of our elite candidate repurposed drugs and detecting the pathways they are involved, as well as by repeating the process using data from 2016, some candidate repurposed drugs show some relevance to AD, creating potential interest for these compounds to be further tested with *in vivo* and clinical experiments. Pathways detected through the candidate repurposed drug targets showed relevance with already known AD pathways using *a priori* knowledge from DisGeNET. We expect that our proposed candidate repurposed drugs will serve as prime candidates for future AD research and our network-based drug repurposing method will be useful for future drug-repurposing studies on many other diseases, beyond AD.

CRediT authorship contribution statement

Kyriaki Savva: Conceptualization, Methodology, Software, Validation, Investigation, Resources, Writing – original draft, Writing – review & editing. **Margarita Zachariou:** Conceptualization, Writing – review & editing, Supervision. **Marilena Bourdakou:** Conceptualization, Writing – review & editing, Supervision. **Nikolas Dietis:** Conceptualization, Writing – review & editing. **George M. Spyrou:** Conceptualization, Writing – review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This research was supported by the Muscular Dystrophy Association Cyprus/ Telethon Cyprus and the European Commission Research Executive Agency (REA) under Grant BIORISE 669026, under the Spreading Excellence, Widening Participation, Science with and for Society Framework.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.csbj.2022.03.013.

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