



# Drug repurposing on Alzheimer's disease through modulation of NRF2 neighborhood

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## ABSTRACT

Alzheimer's disease (AD) is an age-dependent neurodegenerative disorder and the most common cause of cognitive decline. The alarming epidemiological features of Alzheimer's disease, combined with the high failure rate of candidate drugs tested in the preclinical phase, impose more intense investigations for new curative treatments. NRF2 (Nuclear factor-erythroid factor 2-related factor 2) plays a critical role in the inflammatory response and in the cellular redox homeostasis and provides cytoprotection in several diseases including those in the neurodegeneration spectrum. These roles suggest that NRF2 and its directly associated proteins may be novel attractive therapeutic targets in the fight against AD. In this study, through a systemics perspective, we propose an *in silico* drug repurposing approach for AD, based on the NRF2 interactome and regulome, with the aim of highlighting possible repurposed drugs for AD. Using publicly available information based on differential expressions of the NRF2-neighborhood in AD and through a computational drug repurposing pipeline, we derived to a short list of candidate repurposed drugs and small molecules that affect the expression levels of the majority of NRF2-partners. The relevance of these findings was assessed in a four-step computational meta-analysis including i) structural similarity comparisons with currently ongoing NRF2-related drugs in clinical trials ii) evaluation based on the NRF2-diseasome iii) comparison of relevance between targeted pathways of shortlisted drugs and NRF2-related drugs in clinical trials and iv) further comparison with existing knowledge on AD and NRF2-related drugs in clinical trials based on their known modes of action. Overall, our analysis yielded in 5 candidate repurposed drugs for AD. In cell culture, these 5 candidates activated a luciferase reporter for NRF2 activity and in hippocampus derived TH22 cells they increased NRF2 protein levels and the NRF2 transcriptional signatures as determined by increased expression of its downstream target heme oxygenase 1. We expect that our proposed candidate repurposed drugs will be useful for further research and clinical translation for AD.

## 1. Introduction

Alzheimer's disease (AD) is an age dependent progressive neurodegenerative disease and is considered as the leading cause of dementia. Neurodegenerative disorders are characterized by the progressive loss of neurons that are critically involved in learning, thinking and remembering (<https://www.nia.nih.gov/health/alzheimers-disease-fact-sheet>). These disorders are affecting ~55 million people worldwide with an estimation to reach more than 139 million people in 2050 (<https://www.alzint.org/about/dementia-facts-figures/dementia-statistics/>).

AD is defined by memory impairment, cognitive decline and alterations in thinking and behavior. It is characterized by unique pathological changes such as abnormal aggregation of amyloid  $\beta$ -peptide (A $\beta$ ), and hyperphosphorylated tau protein (p-tau) accumulation leading to neuroinflammation and oxidative stress [1]. Despite the devastating effects of AD, its pathogenesis is not fully understood and therapeutic methods to halt its progression remain a challenge [2].

In recent years, many small molecules have been developed for the treatment of AD; however, there are still no drugs with effective disease-modifying effects [3]. Drug repurposing (DR) is an alternative method of

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drug discovery which aims to detect novel indications for existing drugs in order to treat new diseases. DR can be carried out using either experimental methods or computational techniques, the latter of which is commonly known as ‘*in silico* drug repurposing’ [4].

Various *in silico* DR approaches have been developed and could be classified into 5 general categories: knowledge-based, transcriptomic-based, chemical structure-based, network-based, machine learning and data mining-based [5]. In the first category, knowledge-based DR approaches focus on target-based, pathway-based, and target mechanism-based investigations. Within the transcriptomic-based drug repurposing category, gene signatures derived from disease-related omics data are employed, searching for inverse drug-disease relationships. By examining the similarities or dissimilarities in gene expression patterns, potential drug-disease associations can be identified [6]. The chemical structure of drugs is also a valuable resource for identifying potential similarities in transcriptional responses between drugs, which can help to identify opportunities for drug repositioning. Chemical structure similarity can be assessed using various methods, including two-dimensional (2D) topological fingerprints and three-dimensional (3D) conformational fingerprints. These techniques allow the comparison of structural features and facilitate the identification of potential relationships between drugs based on their chemical properties [7,8]. By leveraging network-based approaches, researchers can gain insight into the complex relationships and interactions between drugs, targets, diseases, and other relevant factors, facilitating the identification of potential DR opportunities [9,10]. In addition, the exponential increase in publicly available biological and biomedical data has greatly contributed to the development, exploration, and application of machine learning/deep learning (ML/DL) techniques in the discovery of new drug-disease associations and drug repositioning. The ML/DL algorithms are used for tasks such as binary classification, multi-class classification, and value prediction. By using machine learning models and analyzing large datasets, researchers can uncover hidden patterns and relationships between drugs and diseases, enabling the identification of novel drug-disease associations and potential repositioning applications [11,12].

In the context of identifying potential repurposed drugs for AD, different approaches from the aforementioned categories have been employed. Xie et al. (2016) employed *in silico* drug repurposing approaches from two categories: transcriptomic-based and chemical structure-based to identify repurposed drugs for AD [3]. In addition, network-based drug repurposing approaches have been used to identify candidate drugs for AD [13,14]. Siavelis et al. (2016) applied three different transcriptomic-based drug repurposing tools and proposed a list of 27 potential anti-AD drugs [15].

Recent evidence suggests that Nuclear factor erythroid 2-related factor 2 (NRF2) can prevent some early pathogenic processes in AD. NRF2 is the product of the *NFE2L2* gene and it is a central regulator that protects cells against oxidative, inflammatory or proteotoxic stress [16]. It belongs to the cap'n'collar (CNC) basic-region leucine zipper transcription factor family [17]. NRF2 regulates the expression of about 250 genes that contain a regulatory enhancer called Antioxidant Response Element (ARE). These genes are involved in processes such as in endobiotic and xenobiotic biotransformation reactions, antioxidant metabolism, protein degradation and inflammation. Based on this regulatory network, NRF2 has the role of moderator of the response to various forms of stress [17–20]. Furthermore, NRF2 interacts with numerous other proteins and pathways, providing a complicated regulatory network for several cellular processes [21]. It has been reported that high levels of NRF2 could improve the damages caused by ROS and could be used as a preventer in many AD pathogenic processes [20,22]. Haplotypes of the *NFE2L2* gene promoter have been associated with the early stage of AD, suggesting that variants of the *NFE2L2* gene might affect the progression of AD [23]. The NRF2 interactome and regulome represent very promising pharmacological targets to control common pathological mechanisms behind several diseases and may facilitate the

development of effective NRF2-based therapeutics [24,25]. However, to the best of our knowledge, there are no computational studies that have focused on NRF2 neighborhood to facilitate *in silico* DR approaches, suggesting new therapeutic options for AD.

In this study, we have developed a computational drug repurposing approach that focuses on leveraging the NRF2 neighborhood, including its interactome and regulome, to identify potential candidate drugs for repurposing in AD. To achieve this, we used a transcriptomic-based drug repurposing tool to highlight the drugs with potential relevance. Subsequently, we utilized chemical structure-based and knowledge-based approaches to computationally evaluate these identified drugs. We first explored the differential expression of NRF2 and its partners in AD using publicly available information from transcriptomic data. Using an *in silico* DR pipeline, we highlighted candidate repurposed drugs that were experimentally found to significantly alter the expression of the majority of the NRF2 neighborhood. Our computational approach resulted in 34 shortlisted candidate repurposed drugs which were further analyzed into a four-step meta-analysis. Specifically, the validity of our shortlisted drugs was explored based on their association with other NRF2-related drugs in ongoing clinical trials in the domains of structural similarity, mode of action and pathway targets as well as based on the results of a knowledge-based DR approach that integrates biomedical studies to highlight significant candidate drugs for AD. After conducting the meta-analysis, we found that five candidate drugs, namely curcumin, trichostatin-a, panobinostat, parthenolide, and entinostat, were consistently significant across all steps of the analysis. To assess their potential to activate the NRF2, the five candidate drugs were experimentally tested in cell cultures where all of them activated a luciferase reporter for NRF2 activity and in hippocampus-derived TH22 cells they increased NRF2 protein levels and the NRF2 transcriptional signatures as determined by increased expression of its downstream target heme oxygenase 1.

## 2. Methods

### 2.1. NRF2-interaction partners

The NRF2 neighborhood was obtained from (<http://sbi.imim.es/d/ata/nrf2/>) [26]. More specifically, the authors of this study collected and organized information on physical interactions between proteins involved in the NRF2 regulatory pathway. This information was obtained from the human interactome, which combines data from several sources on protein-protein interactions [27,28]. However, due to the short half-life of the NRF2 protein, some interactions may not have been detected. Nevertheless, they identified several known proteins that interact with NRF2, including KEAP1,  $\beta$ -TrCP, and MAFs. They have also found a group of nuclear proteins involved in the regulation of gene expression that interact with NRF2. In addition, NRF2 is expected to interact with several kinases, such as GSK-3 and several protein kinase C isoforms. They have also identified several biological processes enriched in the NRF2 neighborhood, including metabolic processes related to biosynthesis of pentose, tetrapyrrole, heme, glucose 6-phosphate, cysteine, GSH, glyceraldehyde-3-phosphate, and NADPH. Although these regulatory proteins may not interact directly with each other, they are connected through mediator proteins [26].

### 2.2. Differential expression of NRF2-neighborhood in AD

We investigated the differential expression of NRF2 and its partners in AD using the Expression Atlas - EMBL-EBI database (<https://www.ebi.ac.uk/gxa/home>) (accessed on August 2022) [29]. Expression Atlas is an open resource that allows users to find information about gene and protein expression under specific conditions. We queried genes from the NRF2 neighborhood to find how their expression changes in AD. We filtered out the results keeping those from the experiment with accession number E-GEOD-5281- “Microarray analysis of six brain areas from

Alzheimer's disease patients and normal individuals" [30]. According to the documentation given from Expression Atlas, the dataset was normalized using RMA *oligo* version 1.36.1. The overall analysis was performed in the R statistical environment (<http://www.R-project.org/>) from the Expression Atlas. The dataset was processed using the R package Limma version 3.28.21 [31], a linear model that calculates a moderated t-statistic from gene expression experiments. Independent filtering was performed using *genefilter* version 1.54.2 with gene variances as filter statistic. Adjusted p-values were calculated using Benjamini & Hochberg FDR correction. Differentially expressed genes (DEGs) were selected based on  $|\log_2FC| \geq 1$  with adj. p-values < 0.05. Finally DEGs that maintained their differential expression across all brain areas were selected for further analysis.

### 2.3. Computational drug repurposing

For the case of *in silico* drug repurposing we used a computational tool called Drug Gene Budger (DGB) (<https://maayanlab.cloud/DGB/>) (accessed on August 2022) [32]. DGB is a web based application tool that utilizes large collections of drug - induced transcriptomic signatures to prioritize drugs and small molecules that maximally influence the expression of a target gene. Users can input a gene symbol and specify whether they wish to up-regulate or down-regulate its expression. DGB then presents a prioritized list of small molecules that have been experimentally validated to induce the desired change in gene expression. For each small molecule, DGB provides information such as log-transformed fold change, p-value, and q-value, which quantify the extent of gene expression modification. These values indicate the significance of differential expression as determined by the Limma method. The experimental data used by DGB is extracted from the LINCS L1000 [33] dataset, the original Connectivity Map (CMap) [34] dataset, and the Gene Expression Omnibus (GEO) [35]. In this study we kept the significant small molecules with q-value < 0.05 and absolute  $\log_2FC \geq 1$  that were found to reverse the expression of the selected genes in L1000 data.

### 2.4. Collection of clinical trial drugs with NRF2 activation ability

Firstly, we collected clinical trial drugs for AD with NRF2 activation ability from Osama et al., 2020. Specifically, we collected a) 13 drugs that have been enrolled in AD clinical trials and have been demonstrated

to activate NRF2 (Table 1) and b) 11 NRF2 activators that have been enrolled in different clinical trials and they are showing positive effects on AD in-vivo models (Table 2).

### 2.5. Structural similarity

We downloaded the structures of the candidate repurposed drugs and of the NRF2-related drugs in currently running clinical trials in the form of the Simplified Molecular Input Line Entry Systems (SMILES) from PubChem (<https://pubchem.ncbi.nlm.nih.gov/>) [36]. We then converted the SMILES into a single structure data file (SDF) library file using the OpenBabel software [37]. We used the merged SDF file as input in the ChemBioServer 2.0 (<https://chembioserver.vi-seem.eu/>) [7], a publicly available tool that provides filtering, clustering, comparison of drug structures and networking of chemical compounds to facilitate both drug discovery and repurposing. Drugs were clustered using the Soergel distance  $\leq 0.15$  corresponding to a Tanimoto similarity 87% (Tanimoto similarity =  $1/(1 + \text{Soergel distance})$ ) [38].

### 2.6. ADMET prediction

The *in silico* absorption, distribution, metabolism, excretion, and toxicity (ADMET) properties of the resulted compounds were estimated using AdmetSAR web application [39].

### 2.7. Cell culture and reagents

MCF-7 c32 ARE-LUC and HT22 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, HyClone, CH30160.03) with 80 µg/ml gentamicin (Normon Laboratories). The Curcumin, Etinostat, Panobinostat, Tricostatin-a, Parthenolide and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) were purchased from Sigma Aldrich. All compounds were dissolved in dimethylsulfoxide (DMSO). The final concentration of DMSO in cell culture was less than 0.2%.

### 2.8. Luciferase assay

MCF-7c32 ARE-LUC [40] cells were seeded on 24-well plates (75,000 cells per well) and incubated with each compound at the indicated concentrations for 16 h. Then, cells were lysed and assayed with a luciferase assay system (Promega) according to the manufacturer's

**Table 1**  
Drugs with NRF2-activation ability in AD clinical trials.

Compound	CT ID	Smiles	Clinical_Phase	MoA
<b>DL-3-n-butylphthalide</b>	NCT02711683	<chem>O=C1C2=CC=CC=C2C(CCCC)O1</chem>	Not Applicable	
<b>Resveratrol</b>	NCT01504854	<chem>C1=CC(=CC=C1C=CC2=CC(=CC(=C2)O)O)O</chem>	Phase 2	
<b>Curcumin</b>	NCT00164749	<chem>COC1=C(C=CC(=C1)C=CC(=O)CC(=O)C=CC2=CC(=C(C=C2)O)OC)O</chem>	Phase 1/2	Cyclooxygenase inhibitor histone acetyltransferase inhibitor lipoxygenase inhibitor NFkB pathway inhibitor
<b>Sulforaphane</b>	NCT04213391	<chem>CS(=O)CCCCN=C=S</chem>	Not Applicable	anticancer agent aryl hydrocarbon receptor antagonist
<b>Lipoic Acid</b>	NCT01058941	<chem>C1CSSC1CCCCC(=O)O</chem>	Phase 1/2	reducing agent
<b>Perindopril</b>	NCT02085265	<chem>CCCC(C(=O)OCC)NC(C)(C(=O)N1C2CCCC2CC1C(=O)O</chem>	Phase 2	angiotensin converting enzyme inhibitor
<b>S-Equol</b>	NCT03101085	<chem>C1C(COC2=C1C=CC(=C2)O)C3=CC=C(C=C3)O</chem>	Phase 1/2	estrogen receptor agonist
<b>Doxycycline</b>	NCT00439166	<chem>CC1C2C(C3C(C(=O)C(=C3(C(=O)C2=C(C4=C1C=CC(=C4O)O)O)C(=O)N)N(C)C)O</chem>	Phase 3	bacterial 30S ribosomal subunit inhibitor metalloproteinase inhibitor
<b>Quercetin</b>	NCT04063124	<chem>C1=CC(=C(C=C1C2=C(C(=O)C3=C(C=C(C=C3O2)O)O)O)O</chem>	Phase 1/2	polar auxin transport inhibitor
<b>Genistein</b>	NCT01982578	<chem>C1=CC(=CC=C1C2=COC3=CC(=CC(=C3C2=O)O)O</chem>	Not Applicable	tyrosine kinase inhibitor
<b>Tideglusib</b>	NCT00948259	<chem>C1=CC=C(C=C1)CN2C(=O)N(SC2=O)C3=CC=CC4=CC=CC=C43</chem>	Phase 1/2	glycogen synthase kinase inhibitor
<b>Pyridoxine</b>	NCT00056225	<chem>CC1=NC=C(C(=C1O)CO)CO</chem>	Phase 3	vitamin B
<b>Benfotiamine</b>	NCT02292238	<chem>CC1=NC=C(C(=N1)N)CN(C=O)C(=C(C(COP(=O)(O)O)O)SC(=O)C2=CC=CC=C2)C</chem>	Phase 2	antioxidant

**Table 2**

Drugs with NRF2 activation ability in different clinical trials that are showing positive effects on AD in-vivo models.

Compound	CT ID	Disease	Smiles	Clinical_Phase	MoA
<b>Dimethyl fumarate</b>	NCT01930708	Multiple sclerosis/ Psoriasis	<chem>COC(=O)C=CC(=O)OC</chem>	Phase 4	nuclear factor erythroid derived like (NRF2) activator
<b>Artemether</b>	NCT02089841	Plasmodium falciparum malaria	<chem>CC1CCC2C(C(OC3C2C4CCC(O3)(OO4)C)OC)C</chem>	Phase 4	antimalarial agent
<b>Ginsenoside-Rd</b>	NCT00815763	Ischemic stroke	<chem>CC(=CCCC(C)(C1CCC2(C1C(CC3C2(CC(C4C3(CCC(C4(C(C)O)C)OC5C(C(C(C(O5)CO)O)O)OC6C(C(C(C(O6)CO)O)O)C)O)C)OC7C(C(C(C(O7)CO)O)O)C</chem>	Phase 3	calcium channel blocker
<b>Astaxanthin</b>	NCT03945526	Cerebral stroke	<chem>CC1=C(C(C(C(C1=O)O)(C)C)C=CC(=CC=CC(=CC=CC=C(C)C=CC=C(C)C=CC2=C(C(=O)C(CC2(C)C)O)C)C</chem>	Phase 1	antioxidant
<b>Apigenin</b>	NCT04114916	Cholesterol	<chem>C1=CC(=CC=C1C2=CC(=O)C3=C(C=C(C=C3O2)O)O</chem>	Not applicable	casein kinase inhibitor cell proliferation inhibitor
<b>Hesperidin</b>	NCT04452799	COVID-19	<chem>CC1C(C(C(C(O1)OCC2C(C(C(C(O2)OC3=CC(=C4C(=O)CC(OC4=C3)C5=CC(=C(C=C5)OC)O)O)O)O)O)O</chem>	Early Phase 1	flavanone glycoside
<b>Ebselen</b>	NCT03013400	Bipolar disorder	<chem>C1=CC=C(C=C1)N2C(=O)C3=CC=CC=C3[Se]2</chem>	Phase 2	Cyclooxygenase inhibitor glutathione peroxidase agonist H+/K + -ATPase inhibitor nitric oxide synthase inhibitor
<b>Puerarin</b>	NCT02254655/ NCT00854724	Rheumatoid arthritis/ Alcohol Abuse	<chem>C1=CC(=CC=C1C2=COC3=C(C2=O)C=CC(=C3C4C(C(C(C(O4)CO)O)O)O)O</chem>	Phase 2/Phase 2	serotonin receptor antagonist
<b>Antroquinonol</b>	NCT03622463/ NCT02047344	Atopic dermatitis/Non-small cell lung cancer stage IV	<chem>CC1C(C(C(C(C1=O)OC)OC)O)CC=C(C)CCC=C(C)CCC=C(C)C</chem>	Phase 2/Phase 2	
<b>Vanillic acid</b>	NCT01468259	Renal disease	<chem>COC1=C(C=CC(=C1)C(=O)O)O</chem>	Phase 1	
<b>Allicin</b>	NCT00200785	Arteriosclerosis	<chem>C=CCSS(=O)CC=C</chem>	Not Applicable	cytokine production inhibitor

instructions. Relative light units were measured in a GloMax 96 microplate luminometer with dual injectors (Promega). Each value corresponds to at least three independent samples.

## 2.9. Cell viability assessed by MTT reduction

In live cells but not in dead ones, the tetrazolium ring of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) can be reduced by active dehydrogenases to produce a formazan precipitate. After exposure to the independent drugs for 16h, cells were washed three times with phosphate-buffered saline (PBS) followed by the addition of MTT (0.125 mg/ml) and incubation for 1–2 h at 37 °C. Thereafter, the media was removed and DMSO was added to each well to dissolve the formazan precipitate for 20 min, thereby determining the relative number of alive cells. An aliquot (100 µl) of the supernatants were analyzed in 96-well multiwell plates at 550 nm in a VERSAmax microplate reader (Molecular Devices).

## 2.10. Immunoblotting

This method was performed essentially as described in [41]. Briefly, cells were homogenized in lysis buffer (50 mM TRIS pH 7.6, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 1% SDS), denatured at 95 °C for 15 min, sonicated, and pre-cleared by centrifugation. Twenty µg of protein lysate were resolved in SDS-PAGE, transferred to Immobilon-P (Millipore) membranes and proteins of interest were detected with the following primary antibodies: NRF2 (homemade, validated in [42]), HO-1 (homemade, validated in [43]) and Vinculin (E1E9V, Cell Signaling Technology). Proper peroxidase-conjugated secondary antibodies were used for detection by enhanced chemiluminescence (GE Healthcare).

## 2.11. Statistical analysis

Unless otherwise indicated, all experiments were performed at least 3 times and all data presented in the graphs are the mean of at least 3 independent samples. Data are presented as mean ± S.D. (standard

deviation). Statistical differences between groups were assessed using GraphPad Prism 8 software by the unpaired Student's t-test. One-way analyses of variance with post-Bonferroni's test were used for multiple comparisons. Statistically significant differences are indicated in the figures (\*\*\*) indicate p values < 0.001, \*\* <0.01 and \* <0.05).

## 3. Results

### 3.1. NRF2- related differentially expressed genes found in AD

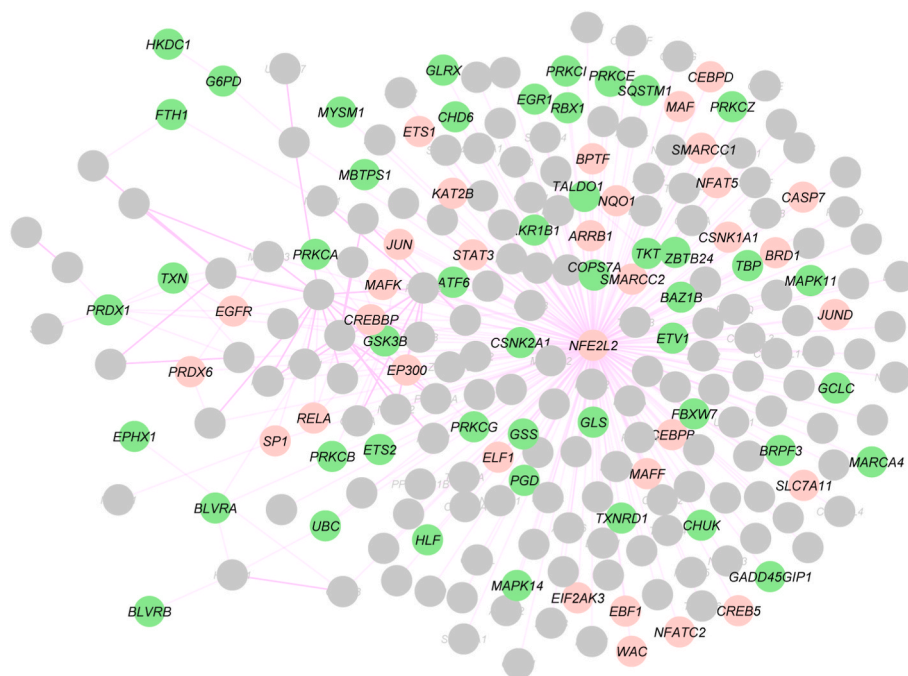
The first part of this study included the identification of the differential expression of the *NFE2L2* and its neighbors. *NFE2L2* and its partners were used as input to the Expression Atlas database to identify those that have been found as differentially expressed in AD. From the 229 genes, 80 were found as differentially expressed in the six brain areas from Alzheimer's disease patients compared to normal individuals. Of these, 47 were found to be under-expressed and 33 were found to be over-expressed including *NFE2L2* (Fig. 1).

### 3.2. Identification of the shortlisted NRF2 – related candidate repurposed drugs for AD

The 80 differentially expressed genes (DEGs) were used separately as input to the DGB drug repurposing tool. Using q-value <0.05 and the absolute log2FC ≥ 1 as filtering criteria, we found candidate drugs that regulate 43 out of 47 under-expressed genes and 29 out of 33 over-expressed genes. Using a majority voting approach we prioritized the candidate drugs based on the number of DEGs they affected. Finally we selected the candidate drugs that reverse the expression of at least the 50% of the DEGs and we ended up with 34 unique candidate repurposed drugs, as shown in Fig. 2. Chemical information and properties of the 34 candidate drugs are presented in Supplementary Table 1.

We found that most of the DEGs are regulated from trichostatin-a (66 associated DEGs) and vorinostat (65 associated DEGs) followed by geldanamycin (51 associated DEGs) and apicidin (50 associated DEGs). Then, WZ-3105 (49 associated DEGs), CGP-60474 (48 associated DEGs), wortmannin (47 associated DEGs), panobinostat and AT-7519 (46





**Fig. 1.** NRF2 interactome and regulome. Over-expressed DEGs are represented with red color and under-expressed with green color. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

associated DEGs), entinostat and alvocidib (45 associated DEGs), daunorubicin (43 associated DEGs), PF-562271 and A-443644 (42 associated DEGs), scriptaid and AS-601245 (41 associated DEGs), tozasertib and BMS-345541 (40 associated DEGs) KM-00927, withaferin-a, BRD-K92317137, parthenolide and ISOX (39 associated DEGs), tubastatin-a, HC-toxin, curcumin, THM-I-94 and PHA-793887 (38 associated DEGs), tacedinaline, PIK-75 and torin-2 (37 associated DEGs) and MLN-4924, triptolide and idarubicin (36 associated DEGs).

### 3.3. Computational insights on molecular mechanisms and mode of actions of the shortlisted repurposed drugs

To further explore the candidate drugs we searched the corresponding gene targets of each drug in several databases such as DrugBank (<https://go.drugbank.com/>) [44], CLUE – The Drug Repurposing Hub (<https://clue.io/repurposing>) [45] and Pubchem as well as in the literature. We then used the web tool Enrichr, using the KEGG 2021 HUMAN version (<https://maayanlab.cloud/Enrichr/>) [46], to determine which pathways the target genes of each drug candidate were involved in. Due to the limited number of drug targets present in many candidate drugs, we did not impose a threshold for pathway selection and we performed a membership analysis. Finally, we selected all pathways that are targeted by each drug.

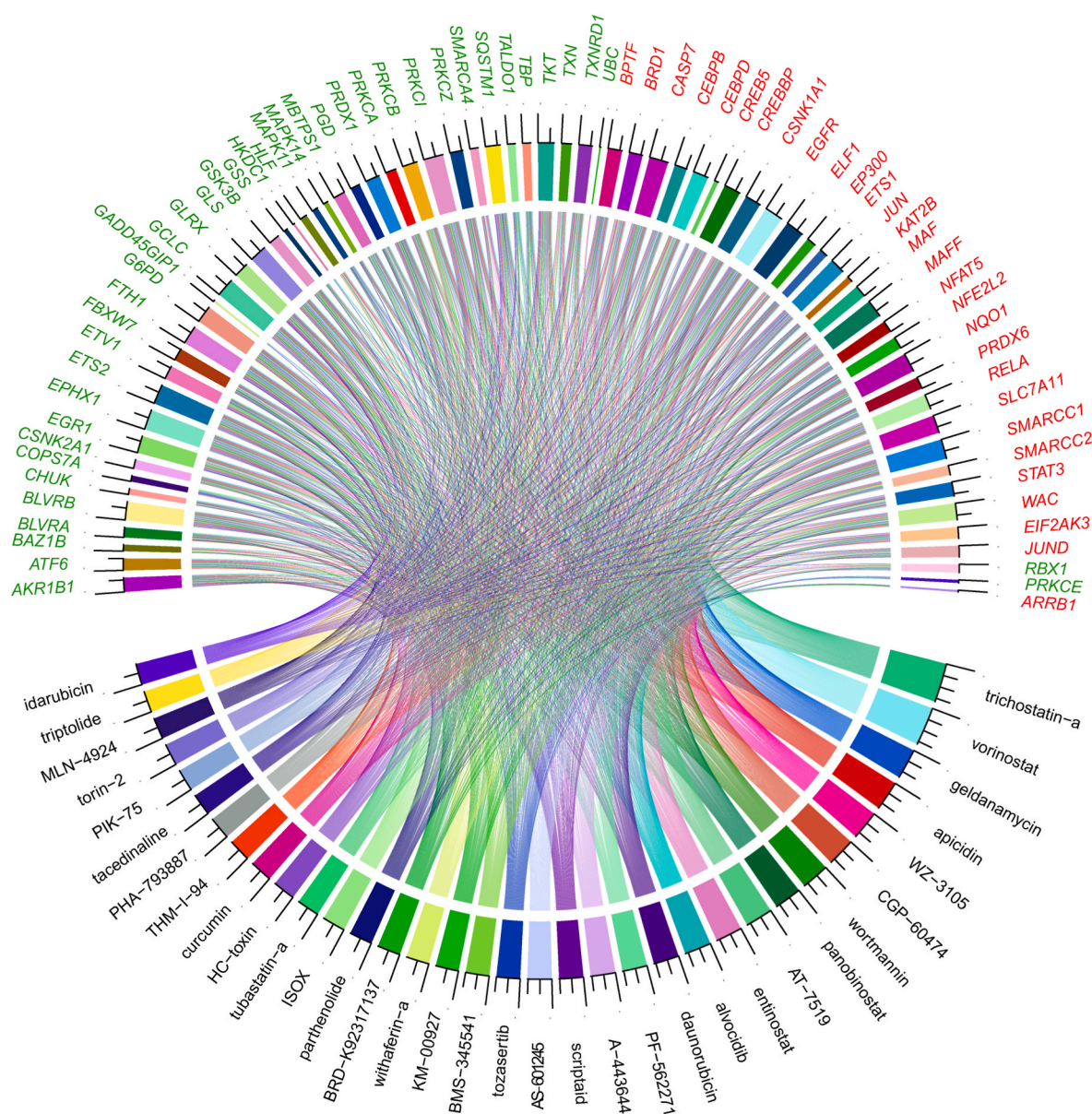
In a similar manner to the selection of candidate drugs, we applied a majority voting approach to select the top pathways that are targeted from at least ~30% of the candidate drugs (Fig. 3A). Specifically, the most targeted pathway is the Pathway in cancer that is targeted from 22 out of 34 drugs followed by Human papillomavirus infection and Viral carcinogenesis that are targeted by 21 and 20 drugs respectively. Moreover, Epstein-Barr virus infection is targeted by 18 drugs, Neutrophil extracellular trap formation and Chronic myeloid leukemia are targeted by 17 drugs, MicroRNAs in cancer by 16 drugs, etc. The whole list of targeted KEGG pathways is presented in **Supplementary File 2**.

Following the same procedure as for the KEGG analysis, we also investigated the Reactome pathways [47] that are targeted by the highlighted repurposed drugs using the version Reactome 2022 in Enrichr. After applying the majority voting approach (Fig. 3B), we found that the most targeted Reactome pathways are Disease and Signal

Transduction, targeted by 26 out of the 34 drugs followed by Gene Expression (Transcription) and Metabolism Of Proteins that are targeted by 22 drugs. Moreover, Generic Transcription Pathway, RNA Polymerase II Transcription and Post-translational Protein Modification are targeted by 21 drugs, Diseases Of Signal Transduction By Growth Factor Receptors And Second Messengers and Signaling By Receptor Tyrosine Kinases by 20 drugs, Infectious Disease, Cell Cycle- Mitotic, Hemostasis and Cell Cycle by 19 drugs, Signaling By Nuclear Receptors, Cellular Responses To Stress and Cellular Responses To Stimuli and Developmental Biology by 18 drugs. The list with all targeted Reactome pathways is also provided in **Supplementary File 2**.

KEGG and Reactome pathway enrichment analyses were also performed to identify the statistically significant enriched pathways involving the 80 DEGs. Using adj. p-value $<0.05$  as selection criterion, we found 166 significant KEGG pathways and 275 significant Reactome pathways (**Supplementary File 3**). The top 20 pathways both from KEGG and Reactome analysis based on their adj. p-values were used to detect common pathways with the most targeted pathways by our shortlisted drugs. For the case of KEGG pathways, 11 out of 20 pathways were found to be common and specifically, Hepatitis B, Pathways in cancer, Lipid and atherosclerosis, Human cytomegalovirus infection, AGE-RAGE signaling pathway in diabetic complications, HIF-1 signaling pathway, Human T-cell leukemia virus 1 infection, PD-L1 expression and PD-1 checkpoint pathway in cancer, Fluid shear stress and atherosclerosis, Shigellosis and Kaposi sarcoma-associated herpesvirus infection. For the case of Reactome pathways, 15 out of 20 pathways were found to be common: Disease, Gene Expression (Transcription), Generic Transcription Pathway, RNA Polymerase II Transcription, Diseases Of Signal Transduction By Growth Factor Receptors And Second Messengers, Cellular Responses To Stress, Cellular Responses To Stimuli, Transcriptional Regulation By TP53, Signaling By NOTCH, NOTCH1 Intracellular Domain Regulates Transcription, Constitutive Signaling By NOTCH1 HD + PEST Domain Mutants, Signaling By NOTCH1, Transcriptional Regulation By RUNX1, Signaling By Interleukins and Cellular Response To Chemical Stress.

We also investigated the available modes of action (MoAs) of the 34 shortlisted repurposed drugs using information from the CLUE – The Drug Repurposing Hub. Using a majority voting approach, we found that



**Fig. 2.** Circos plot that summarizes the associations between the shortlisted repurposed drugs and the NRF2-related DEGs. Over-expressed DEGs are represented with red color and under-expressed with green color. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

our shortlist is enriched for HDAC inhibitors - eleven in total - followed by four CDK inhibitors, three NF $\kappa$ B pathway inhibitors and two PI3K and Topoisomerase inhibitors. The MoAs of the remaining repurposed drugs belong to the following categories: AKT inhibitor, Apoptosis stimulant, Aurora kinase inhibitor, Bcr-Abl kinase inhibitor, Cell cycle inhibitor, Cyclooxygenase inhibitor, DNA protein kinase inhibitor, FLT3 inhibitor, Focal adhesion kinase inhibitor, Histone acetyltransferase inhibitor, HSP inhibitor, IKK inhibitor, JAK inhibitor, JNK inhibitor, Lipoxigenase inhibitor, mTOR inhibitor, NEDD8 activating enzyme inhibitor, RNA polymerase inhibitor and RNA synthesis inhibitor (Fig. 4).

### 3.4. Computational validity checks for the shortlisted candidate repurposed drugs

To gain insight into the 34 candidate drugs, we performed a four-step meta-analysis procedure. First, we explored the structural similarity between the candidate repurposed drugs and other NRF2-related drugs that are in clinical trials for AD or that are also in different clinical trials

but show positive effects in AD *in vivo* models (Tables 1 and 2, see Methods). In the second step, we explored the associations between the shortlisted repurposed drugs and the NRF2-diseasome. To further explore the relevance of our findings with already known NRF2-related clinical trial drugs we also compared the molecular mechanisms that they target. In the final step we evaluated our list of drugs based on their modes of action with respect to ongoing clinical trials and we compared them with the findings from an in-house developed pipeline that utilizes *a priori* knowledge from computational drug repurposing studies and clinical trials to evaluate candidate drugs [48].

### i) Investigation of Structural Similarity with respect to ongoing clinical trials

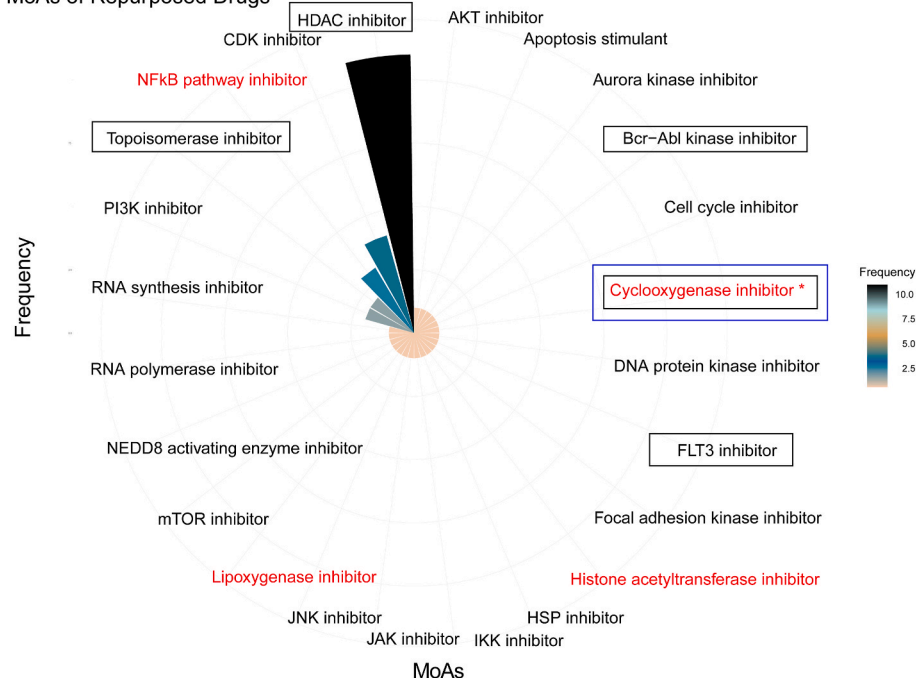
The shortlisted candidate repurposed drugs were screened for structural similarity with the NRF2-related drugs in clinical trials. Pair-wise structural similarity was calculated using a Tanimoto score threshold of 87%. As shown in Fig. 5 the hierarchical clustering suggests



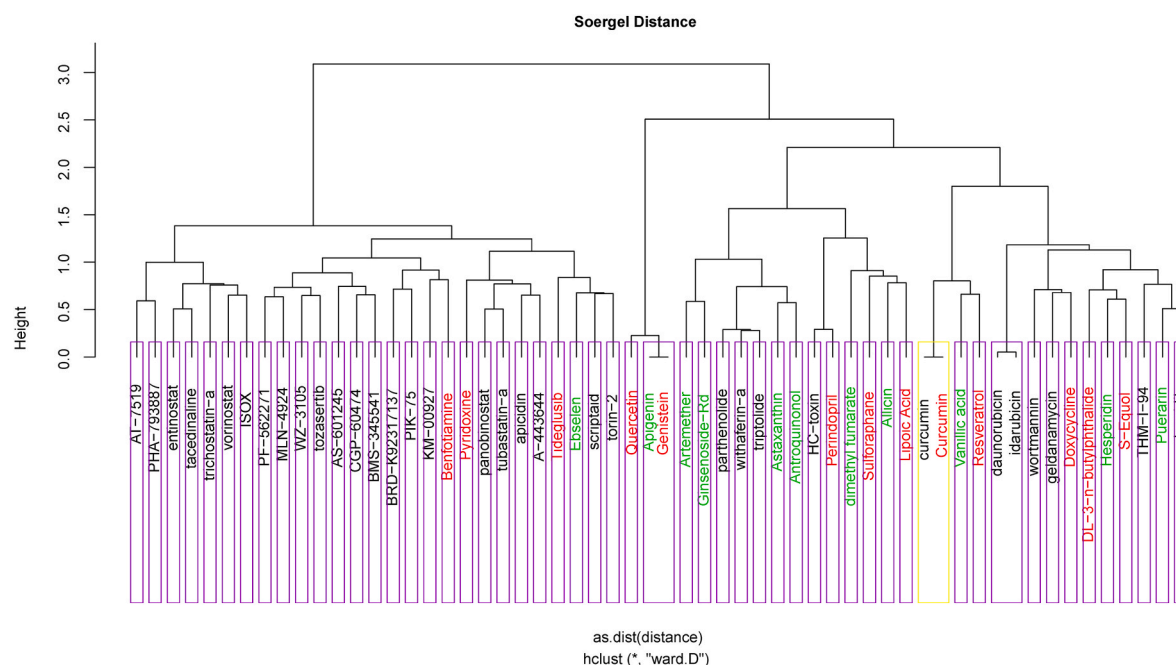


**Fig. 3.** Coord polar plot of the top targeted A) KEGG and B) Reactome pathways of the shortlisted repurposed drugs based on the majority voting. Common pathways with the NRF2-related drugs in AD clinical trials are represented with red color, common pathways with the NRF2-related drugs in other clinical trials with positive effects in AD *in vivo* models with \* and pathways that were also found as significantly enriched from the pathway analysis of the NRF2-related DEGs with rectangular border. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

## MoAs of Repurposed Drugs



**Fig. 4.** Coord polar plot of the MoAs of the short-listed repurposed drugs based on the majority voting. Common MoAs with the NRF2-related drugs in AD clinical trials are represented with red color, common MoAs with the NRF2-related drugs in other clinical trials with positive effects in AD *in vivo* models with \*, MoAs that were also found as significant related with AD from repurposed drug lists with black rectangular border MoAs that were also found as significant related with AD from clinical trial drugs with blue rectangular border. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)



**Fig. 5.** Hierarchical clustering of the shortlisted repurposed drugs along with NRF2-related drugs in ongoing clinical trials based on structure similarity. The different groups in each box are thresholded at Soergel distance value 0.15. The 34 shortlisted repurposed drugs are presented with black color font, the 13 NRF2-related drugs in AD clinical trials with red font and the 11 NRF2-related drugs in other clinical trials with green font. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

that the majority of all drugs are not very similar, indicating a lack of redundancy and a wide range of structural diversity in both our short list and clinical trials. Specifically, the total of 58 drugs (34: shortlisted candidate repurposed drugs-black font, 13 NRF2-related drugs in AD clinical trials – red font, 11 NRF2-related drugs in other clinical trials-green font) were clustered into 55 independent clusters and only three clusters contain more than one drug. One of these three clusters contains the same drug-curcumin – that is currently in an ongoing trial for AD and

it was highlighted as significant repurposed from our analysis. The second cluster contains two NRF2-related drugs, genistein which is currently in an ongoing clinical trial for AD and apigenin, which is in an ongoing clinical trial for cholesterol and has been shown to have beneficial effects on AD *in vivo* models [49]. These two drugs were found to be structurally identical based on their molecular fingerprints. The third cluster contains the only highly similar repurposed compounds from our list (95% Tanimoto similarity), daunorubicin and idarubicin.



## ii) Evaluation of the shortlisted drugs with the NRF2-diseasome

To further analyze and interpret the findings from our DR pipeline the associated diseases of the 34 candidate repurposed drugs were compared with the NRF2-related diseases. Specifically, we searched in the Comparative Toxicogenomics Database –CTD (<http://ctdbase.org/>) [50] for the curated disease associations of our shortlisted drugs. Curated associations are extracted from the published literature by CTD curators. From the associated diseases we then detected the commonalities between the NRF2-diseasome acquired from Cuadrado et al., 2018 [26]. The NRF2-diseasome was developed by curating a list of 37 NRF2-related diseases, followed by the reliability score based on DisGeNET [51] (Table 3). As described in the aforementioned study [26], the curation of the disease list was based on the following criteria: 1) only phenotypes with more than one citation in Pubmed were selected; 2) the threshold of the score of reliability was set to 0.001 and 3) disease similar names were simplified to one single entry. This analysis resulted in 13 repurposed drugs out of 34 that are associated with NRF2-related diseases. As shown in Table 3, **curcumin** is associated with 19 out of 37 NRF2-related diseases, **vorinostat** with 8, **daunorubicin**, **triptolide** and **trichostatin-a** with 5, **alvocidib** and **idarubicin** with 4, **panobinostat** and **parthenolide** with 2 and **wortmannin**, **entinostat**, **as-601245** and **tozasertib** with 1.

## iii) Comparison of the shortlisted candidate repurposed drugs with NRF2-related drugs in clinical trials based on connected pathways

In the third step we compared the molecular mechanisms of our candidate repurposed drugs with those of existing NRF2-related drugs in ongoing clinical trials. We followed the same approach as for our repurposed drugs. Specifically, we detected the corresponding target genes, the related pathways and the MoAs of the NRF2-related clinical

trial drugs from Tables 1 and 2. Using the majority voting approach, we selected the top KEGG and Reactome pathways that are targeted by least ~30% of the NRF2-related drugs in clinical trials.

For the case of KEGG pathways, of the 13 drugs in clinical trials for Alzheimer's disease that have been shown to activate NRF2 (Table 1), 8 target the Pathways in cancer. In addition, Alzheimer disease, Chemical carcinogenesis, Human cytomegalovirus infection, Kaposi sarcoma-associated herpesvirus infection, Lipid and atherosclerosis and Pathways of neurodegeneration are targeted from 5 out of 13 drugs and Diabetic cardiomyopathy, Human papillomavirus infection, IL-17 signaling pathway, MicroRNAs in cancer, Oxytocin signaling pathway, Serotonergic synapse and Thyroid hormone signaling pathway from 4 drugs. In addition Arachidonic acid metabolism, Breast cancer, Chemokine signaling pathway, Cholinergic synapse, C-type lectin receptor signaling pathway, Estrogen signaling pathway, Gastric acid secretion, Leishmaniasis, NF-kappa B signaling pathway, Ovarian steroidogenesis, Platelet activation, Prolactin signaling pathway, Proteoglycans in cancer, Regulation of lipolysis in adipocytes, Retrograde endocannabinoid signaling, Small cell lung cancer, TNF signaling pathway, VEGF signaling pathway and Wnt signaling pathway are targeted by 3 out of the 13 drugs. We also searched the Reactome pathways that are targeted from the NRF2-related drugs in AD clinical trials and we found that 8 out of 13 target the Immune System pathway. Furthermore, Metabolism, Signaling By Interleukins, Cytokine Signaling In Immune System, Disease and Metabolism Of Proteins targeted from 6 out of 13 drugs, Metabolism Of Lipids, Innate Immune System, Signal Transduction from 5 out of 13 drugs, Biological Oxidations, Metabolism Of Water-Soluble Vitamins And Cofactors, Metabolism Of Vitamins And Cofactors, Transcriptional Regulation By RUNX2, Post-translational Protein Modification, Cellular Responses To Stress, Gene Expression (Transcription), Cellular Responses To Stimuli, Cellular Response To Chemical Stress, Generic Transcription Pathway, Diseases Of Signal Transduction By

**Table 3**

NRF2-related diseases from the DisGeNet database with their reliability scores and the corresponding associated shortlisted repurposed drugs from our analysis.

Diseases	Reliability Score	Repurposed Drugs	Diseases	Reliability Score	Repurposed Drugs
Diabetic nephropathy	0.2016	vorinostat, curcumin	Diabetic cardiomyopathy	0.0803	
Liver cirrhosis	0.2005	curcumin	Middle cerebral artery infraction	0.08	
Non-alcoholic steatohepatitis	0.2005		Breast neoplasms	0.0087	vorinostat, wortmannin, alvocidib, parthenolide, curcumin
Acute kidney injury	0.2	trichostatin-a, curcumin, triptolide, idarubicin	Vitiligo	0.0076	
Pulmonary fibrosis	0.2	panobinostat	Atherosclerosis	0.0067	curcumin
Non-small cell lung carcinoma	0.1252	vorinostat, curcumin	Asthma	0.0043	
Squamous cell carcinoma	0.1243	vorinostat, curcumin	Leukemia	0.0038	alvocidib, daunorubicin, curcumin, idarubicin, as-601245, vorinostat, tozasertib, trichostatin-a, triptolide
Liver neoplasms	0.1238	vorinostat	Colon neoplasm	0.0038	trichostatin-a
Hyperglycemia	0.1208	alvocidib, curcumin	Gastrointestinal diseases	0.0029	vorinostat
Drug induced liver injury	0.12	daunorubicin, curcumin, triptolide	Parkinson disease	0.0026	curcumin
Prostatic neoplasms	0.12	trichostatin-a, vorinostat, panobinostat, entinostat, alvocidib, parthenolide, curcumin	Systemic lupus erythematosus nephritis	0.0026	triptolide
Chronic obstructive pulmonary disease	0.0899		Glioma	0.0024	
Colorectal neoplasms	0.0847		Amyotrophic lateral sclerosis	0.0022	
Alzheimer disease	0.0837	curcumin	Ischemia	0.0016	curcumin
Type 2 diabetes mellitus	0.0814	curcumin	Pulmonary emphysema	0.0013	curcumin
Chronic kidney disease	0.0808	daunorubicin, curcumin, triptolide, idarubicin, daunorubicin, curcumin	Pancreatic neoplasms	0.0013	trichostatin-a
Diabetic retinopathy	0.0805		Vascular diseases	0.0013	daunorubicin
Huntington's disease	0.0805		Sepsis	0.0013	curcumin, idarubicin

Growth Factor Receptors And Second Messengers and RNA Polymerase II Transcription from 4 drugs. Additionally, 36 Reactome pathways are also targeted from 3 out of 13 drugs and specifically Synthesis Of Prostaglandins (PG) And Thromboxanes (TX), Arachidonic Acid Metabolism, Phase I - Functionalization Of Compounds, Fatty Acid Metabolism, Biosynthesis Of EPA-derived SPMs, Synthesis Of 15-Eicosatetraenoic Acid Derivatives, Biosynthesis Of DHA-derived SPMs, Nicotinamide Salvaging, Biosynthesis Of Specialized Proresolving Mediators (SPMs), Nicotinate Metabolism, Interleukin-10 Signaling, Interleukin-4 And Interleukin-13 Signaling, SUMO E3 Ligases SUMOylate Target Proteins, SUMOylation, ESR-mediated Signaling, Signaling By Nuclear Receptors, Transcriptional Regulation By AP-2 (TFAP2) Family Of Transcription Factors, Heme Signaling, Extra-nuclear Estrogen Signaling, Nuclear Events Mediated By NFE2L2, Signaling By Receptor Tyrosine Kinases, KEAP1-NFE2L2 Pathway, Estrogen-dependent Gene Expression, Transcriptional Regulation By RUNX1, Deubiquitination, Developmental Biology, Neutrophil Degranulation, Axon Guidance, Cell Cycle- Mitotic, Nervous System Development, Cell Cycle, Infectious Disease, PI3K/AKT Signaling In Cancer, PIP3 Activates AKT Signaling, Intracellular Signaling By Second Messengers, Regulation Of RUNX2 Expression And Activity.

For the case of 11 NRF2 activators that have been enrolled in different clinical trials and they are showing positive effects in AD in-vivo models (Table 2), examining the KEGG pathways, we found that 3 of them target the Pathways in cancer and the cAMP signaling. Furthermore, Hepatocellular carcinoma, Thyroid hormone synthesis, Gastric acid secretion, Bile secretion, Pancreatic secretion, Chronic myeloid leukemia, Small cell lung cancer, Prostate cancer, Measles, Hepatitis C, Influenza A, Kaposi sarcoma-associated herpesvirus infection, Epstein-Barr virus infection, Viral carcinogenesis, Human cytomegalovirus infection and Chemical carcinogenesis are targeted by 2 out of the 11 drugs. Following the same procedure for the Reactome pathways, we also found that 5 out of 11 drugs target the Post-translational Protein Modification Metabolism Of Proteins. Moreover, Ub-specific, Processing Proteases, Deubiquitination, Cellular Responses To Stress, Cellular Responses To Stimuli, Disease, Signal Transduction and Transport Of Small Molecules are targeted by 3 out of the 11 drugs and Cellular Response To Chemical Stress, Adaptive Immune System, Immune System, SUMO E3 Ligases SUMOylate Target Proteins, SUMOylation, Metabolism Of Steroids, Metabolism Of Lipids, Metabolism, RHO GTPase Effectors, Cell Cycle, Mitotic, Vesicle-mediated Transport, Signaling By Rho GTPases, Cell Cycle, Signaling By Rho GTPases, Miro GTPases And RHOBTB3, Generic Transcription Pathway, RNA Polymerase II Transcription and Gene Expression (Transcription) were targeted by 2 of 11 drugs.

As presented in Fig. 3A, ten KEGG pathways are targeted by both repurposed drugs and NRF2-related drugs in ongoing clinical trials for AD. Precisely, Pathways in cancer, Alzheimer disease, Chemical carcinogenesis, Human cytomegalovirus infection, Kaposi sarcoma-associated herpesvirus infection, Lipid and atherosclerosis, Human papillomavirus infection, MicroRNAs in cancer, Thyroid hormone signaling pathway and Small cell lung cancer. These KEGG pathways are targeted by 24 out of the 34 candidate repurposed drugs: A-443644, alvocidib, AT-7519, BMS-345541, CGP-60474, curcumin, entinostat, HC-toxin, panobinostat, parthenolide, PF-562271, PHA-793887, PIK-75, scriptaid, tacedinaline, THM-I-94, torin-2, trichostatin-a, triptolide, vorinostat, wortmannin, geldanamycin, MLN-4924 and apicidin. In addition, 33 Reactome pathways are also targeted by both repurposed drugs and NRF2-related drugs in ongoing clinical trials for AD: Disease, Signal Transduction, Gene Expression (Transcription), Metabolism Of Proteins, Generic Transcription Pathway, RNA Polymerase II Transcription, Post-translational Protein Modification, Diseases Of Signal Transduction By Growth Factor Receptors And Second Messengers, Signaling By Receptor Tyrosine Kinases, Infectious Disease, Cell Cycle-Mitotic, Cell Cycle, Signaling By Nuclear Receptors, Cellular Responses To Stress, Cellular Responses To Stimuli, Developmental Biology, ESR-

mediated Signaling, PIP3 Activates AKT Signaling, Intracellular Signaling By Second Messengers, SUMO E3 Ligases SUMOylate Target Proteins, SUMOylation, Transcriptional Regulation By RUNX2, Estrogen-dependent Gene Expression, Nervous System Development, Metabolism, Transcriptional Regulation By RUNX1, Immune System, Cytokine Signaling In Immune System, Metabolism Of Lipids, Signaling By Interleukins, Innate Immune System, Cellular Response To Chemical Stress and Axon Guidance. These Reactome pathways are targeted by 29 out of the 34 repurposed drugs from our list: trichostatin-a, vorinostat, geldanamycin, apicidin, CGP-60474, wortmannin, panobinostat, AT-7519, entinostat, alvocidib, PF-562271, A-443644, scriptaid, tozasertib, BMS-345541, tubastatin-a, HC-toxin, curcumin, PHA-793887, THM-I-94, tacedinaline, PIK-75, torin-2, triptolide, ISOX, parthenolide, daunorubicin, MLN-4924 and idarubicin.

In addition, 12 common KEGG pathways were found to be shared between candidate repurposed drugs and NRF2-related drugs in ongoing clinical trials for other diseases but with positive effects in AD in-vivo models: Pathways in cancer, Chronic myeloid leukemia, Small cell lung cancer, Prostate cancer, Measles, Hepatitis C, Influenza A, Kaposi sarcoma-associated herpesvirus infection, Epstein-Barr virus infection, Viral carcinogenesis, Human cytomegalovirus infection and Chemical carcinogenesis. 25 out of the 34 shortlisted repurposed drugs target these pathways A-443644, alvocidib, AT-7519, BMS-345541, CGP-60474, curcumin, entinostat, geldanamycin, HC-toxin, panobinostat, parthenolide, PF-562271, PHA-793887, PIK-75, scriptaid, tacedinaline, THM-I-94, torin-2, trichostatin-a, triptolide, vorinostat, wortmannin, apicidin, ISOX and tubastatin-a. On the other hand 18 common Reactome pathways were also found to be shared between candidate repurposed drugs and NRF2-related drugs in ongoing clinical trials for other diseases, Disease, Signal Transduction, Gene Expression (Transcription), Metabolism Of Proteins, Generic Transcription Pathway, RNA Polymerase II Transcription, Post-translational Protein Modification, Cell Cycle, Mitotic, Cell Cycle, Cellular Responses To Stress, Cellular Responses To Stimuli, SUMO E3 Ligases SUMOylate Target Proteins, SUMOylation, Metabolism, Immune System, Metabolism Of Lipids, Adaptive Immune System and Cellular Response To Chemical Stress. The 29 repurposed drugs mentioned above also target these common Reactome pathways.

Five KEGG pathways were found to be common to all lists: Pathways in cancer, Chemical carcinogenesis, Human cytomegalovirus infection, Kaposi sarcoma-associated herpesvirus infection and Small cell lung cancer. These are targeted by 21 out of the 34 drugs from our list A-443644, alvocidib, AT-7519, BMS-345541, CGP-60474, curcumin, entinostat, geldanamycin, HC-toxin, panobinostat, parthenolide, PF-562271, PHA-793887, PIK-75, scriptaid, tacedinaline, THM-I-94, torin-2, trichostatin-a, triptolide and wortmannin. In addition, 17 Reactome pathways were found to be common across all lists: Disease, Signal Transduction, Gene Expression (Transcription), Metabolism Of Proteins, Generic Transcription Pathway, RNA Polymerase II Transcription, Post-translational Protein Modification, Cell Cycle- Mitotic, Cell Cycle, Cellular Responses To Stress, Cellular Responses To Stimuli, SUMO E3 Ligases SUMOylate Target Proteins, SUMOylation, Metabolism, Immune System, Metabolism Of Lipids and Cellular Response To Chemical Stress. These pathways are also targeted by the 29 repurposed drugs mentioned above.

Finally, for the case of KEGG pathways three out of five common pathways that were found to be common across all lists, are also common with the top 20 significantly enriched KEGG pathways: Pathways in cancer, Human cytomegalovirus infection and Kaposi sarcoma-associated herpesvirus infection (Fig. 3A). The aforementioned 21 drugs: **A-443644, alvocidib, AT-7519, BMS-345541, CGP-60474, curcumin, entinostat, geldanamycin, HC-toxin, panobinostat, parthenolide, PF-562271, PHA-793887, PIK-75, scriptaid, tacedinaline, THM-I-94, torin-2, trichostatin-a, triptolide and wortmannin** also target these three common pathways.

Following the same procedure for the case of Reactome pathways, 7

out of the 17 common pathways, were also found in the top 20 significantly enriched Reactome pathways: Disease, Gene Expression (Transcription), Generic Transcription Pathway, RNA Polymerase II Transcription, Cellular Responses To Stress, Cellular Responses To Stimuli, Cellular Response To Chemical Stress (Fig. 3B). These pathways are targeted by 26 repurposed drugs: **trichostatin-a**, **vorinostat**, **geldanamycin**, **apicidin**, **CGP-60474**, **wortmannin**, **panobinostat**, **AT-7519**, **entinostat**, **alvocidib**, **PF-562271**, **A-443644**, **scriptaid**, **tozasertib**, **BMS-345541**, **tubastatin-a**, **HC-toxin**, **curcumin**, **PHA-793887**, **THM-I-94**, **tacedinaline**, **PIK-75**, **torin-2**, **triptolide**, **ISOX** and **parthenolide**.

#### iv) Evaluation of the shortlisted candidate repurposed drugs based on their modes of action

For the next step, common MoAs between the three lists of drugs were found. First, we searched the Drug Repurposing Hub in order to find the MoAs of the NRF2-related drugs in clinical trials. As presented in Fig. 4, four MoAs were found to be common between repurposed drugs and NRF2-related drugs in clinical trials for AD including NfκB pathway inhibitor, Lipoxygenase inhibitor, Histone acetyltransferase inhibitor and Cyclooxygenase Inhibitor which correspond to 3 repurposed drugs from our list withaferin-a, parthenolide and curcumin. Cyclooxygenase Inhibitor was also found to be common MoA between the three drug lists, corresponding to curcumin.

The MoAs of the shortlisted drugs were also compared with results from an in house approach that utilizes *a priori* knowledge from four neurodegenerative diseases (including AD) and specifically from drugs that are associated with them either from previous drug repurposing works or from clinical trials and gives a score based on this information. Specifically, this methodology has generated frequency tables for each disease based on the pathways, initial indications and MoAs of the drugs identified from the computational drug repurposing studies and clinical trials. The frequency tables of MoAs from AD resulting from repurposed drugs and drugs in ongoing clinical trials and specifically MoAs with a score  $\geq 0.3$  were compared with the MoAs of the shortlisted candidate repurposed drugs. As shown in Fig. 4, five MoAs (HDAC inhibitor, Topoisomerase inhibitor, FLT3 inhibitor, Bcr-Abl kinase inhibitor and Cyclooxygenase inhibitor) were found to be common with the highest scoring MoAs of the AD repurposed drugs (grey circles) and one was found to be common between the highest scoring MoAs of the repurposed drugs and the clinical trials (Cyclooxygenase inhibitor). Of our shortlisted candidate repurposed drugs, 13 fall under these MoAs: tozasertib, curcumin, trichostatin-a, vorinostat, apicidin, panobinostat, entinostat, scriptaid, ISOX, tubastatin-a, HC-toxin, tacedinaline, THM-I-94 and idarubicin.

Merging in a union unique list the candidate repurposed drugs from the two approaches we resulted in a short list with 16 drugs: **tozasertib**, **curcumin**, **trichostatin-a**, **vorinostat**, **apicidin**, **panobinostat**, **entinostat**, **scriptaid**, **isox**, **tubastatin-a**, **hc-toxin**, **tacedinaline**, **thm-i-94**, **idarubicin**, **withaferin-a** and **parthenolide**.

Finally, combining the resulted repurposed drugs from all the meta-analysis steps, 5 candidate repurposed drugs were found to be significant by all steps: **curcumin**, **trichostatin-a**, **panobinostat**, **parthenolide** and **entinostat**.

### 3.5. Experimental validation of our findings

We performed a preliminary *in silico* pharmacokinetics (PK) analysis using the AdmetSAR web application and we observed that the predictions for passage through the gastrointestinal (GI) tract and the blood-brain barrier (BBB) were reasonably good for most of the 5 candidates. However, curcumin appeared to have less favorable predictions compared to the other candidates. We also determined whether these 5 molecules activate NRF2 in cell culture. MCF7 cells carrying a NRF2 reporter of luciferase activity were submitted to the indicated

concentrations of the five compounds (Fig. 6A). To different extents, all compounds dose-dependently activated the NRF2 reporter. In parallel samples, we also analyzed toxicity by the MTT method and found that none of them was toxic at the employed concentrations that activate the NRF2 reporter (Fig. 6B). Considering the variability in response among different cell types, and our interest in neuronal protection, we analyzed the effect of the five compounds on the hippocampus-derived HT22 cells (Fig. 7). These cells were maintained in 0.5% serum medium for 16 h and then submitted to 10  $\mu$ M of each drug for another 16 h. We found that all compounds increased NRF2 protein levels, with curcumin and parthenolide having the greatest effect. We also analyzed the levels of the downstream HO-1 whose expression is regulated by NRF2. Again, all compounds increased HO-1 levels. Among the five compounds tested, parthenolide produced the strongest increase in both NRF2 and HO-1 protein levels. Taken together, these results further validate our *in silico* approach.

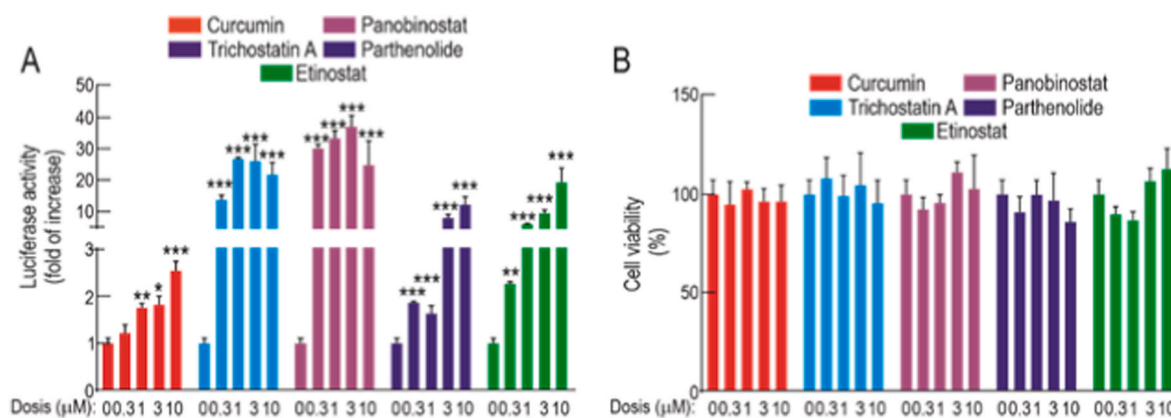
## 4. Discussion

Five drugs have been approved by the FDA for AD clinical treatment. However, none of them can stop the underlying progression of AD but only improve cognitive function. Several studies have investigated the ability of NRF2 to reduce the oxidative stress and the inflammation and some NRF2-activators have shown promising results in AD animal models [20,52,53]. NRF2 activation appears to be a novel and attractive therapeutic approach for AD.

In this study, we present an *in silico* DR methodology for AD focusing on the NRF2 interactome and regulome. By integrating different computational DR approaches from different categories, our goal was to identify and evaluate potential candidate drugs for AD. Using information from the Expression Atlas, our analysis was based on differential expression of the NRF2-neighborhood in AD and through a computational drug repurposing pipeline we found drugs and small molecules that affect the expression levels of these genes. This approach resulted in a total of 34 shortlisted NRF2-related candidate repurposed drugs against AD that alter the expression of the majority of NRF2-partners. Computational insights into the validity of these findings were performed in a four-step meta-analysis including i) structural similarity with current ongoing NRF2-related drugs ii) evaluation based on the NRF2-disease ii) comparison of relevance between targeted pathways of shortlisted drugs and ongoing NRF2-related drugs and iv) further comparison with existing knowledge on AD and current NRF2-related drugs based on mode of actions.

Apart from curcumin which was found to be significant in our analysis and is already in clinical trials for AD, the shortlisted candidate repurposed drugs as well as the NRF2-related drugs in clinical trials were generally shown to be structurally different from each other, meaning that most of the drugs proposed do not belong in the same structural subgroup. By examining the associated diseases of the shortlisted candidate repurposed drugs, we found that 13 drugs from our list are associated with NRF2-related diseases. The two candidate repurposed drugs that are associated with the most NRF2-related diseases are curcumin and vorinostat. Curcumin has the ability to activate the NRF2 by repressing KEAP1 expression [53] and is currently in 7 ongoing clinical trials for AD (NCT01001637, NCT00164749, NCT00099710, NCT01811381, NCT01716637, NCT03761381, and NCT04606420). In addition, a recent clinical trial is investigating the efficacy of vorinostat in patients with mild AD (NCT03056495).

Five KEGG pathways and 17 Reactome pathways were found to be common targets between the candidate repurposed drugs from our list and the ongoing NRF2-related drugs. From these pathways 3 KEGG (Pathways in cancer, Human cytomegalovirus infection and Kaposi sarcoma-associated herpesvirus infection) and 7 Reactome (Disease, Gene Expression (Transcription), Generic Transcription Pathway, RNA Polymerase II Transcription, Cellular Responses To Stress, Cellular Responses To Stimuli and Cellular Response To Chemical Stress) were also



**Fig. 6.** Evaluation of Curcumin, Trichostatin-a, Panobinostat, Parthenolide, and Entinostat ARE activation and cell viability. A) MCF-7 c32 ARE-LUC reporter cells were serum-depleted (<0.5%) for 8 h and then subjected to the indicated concentrations for each compound. 0.1% DMSO was used as vehicle. A, luciferase activity was measured after 16 h of treatment. Data are mean  $\pm$  S.D. (n = 4). \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001 vs. vehicle according to a one-way ANOVA test. B) MTT assay was performed to assess cell viability of each compound in serum-depleted cells. Data are mean  $\pm$  S.D. (n = 4).

found as significantly enriched pathways from the KEGG and Reactome enrichment analysis of the 80 NRF2-related DEGs. Twenty one of the 34 shortlisted drugs, target these three common KEGG pathways A-443644, alvocidib, AT-7519, BMS-345541, CGP-60474, curcumin, entinostat, geldanamycin, HC-toxin, panobinostat, parthenolide, PF-562271, PHA-793887, PIK-75, scriptaid, tacedinaline, THM-I-94, torin-2, trichostatin-a, triptolide and wortmannin and 26 out of 34 compounds trichostatin-a, vorinostat, geldanamycin, apicidin, CGP-60474, wortmannin, panobinostat, AT-7519, entinostat, alvocidib, PF-562271, A-443644, scriptaid, tozasertib, BMS-345541, tubastatin-a, HC-toxin, curcumin, PHA-793887, THM-I-94, tacedinaline, PIK-75, torin-2, triptolide, ISOX and parthenolide target the 7 common Reactome pathways.

In the final step of the meta-analysis we highlighted drugs based on their MoAs. We compared the MoAs of the repurposed drugs with others from existing knowledge on AD and from NRF2-related drugs in ongoing trials and we concluded to 16 drugs including tozasertib, curcumin, trichostatin-a, vorinostat, apicidin, panobinostat, entinostat, scriptaid, isox, tubastatin-a, hc-toxin, tacedinaline, thm-i-94, idarubicin, withaferin-a and parthenolide. Tubastatin-a is an HDAC inhibitor and has been shown to have a therapeutic effect in reducing tau pathology in animal models of AD [54,55].

Combining the results of the above meta-analysis steps, five candidate repurposed drugs were found to be significant by all steps curcumin, trichostatin-a, panobinostat, parthenolide and entinostat. Three of these drugs (trichostatin-a, panobinostat and entinostat) are histone deacetylases (HDAC) inhibitors. HDAC inhibitors have shown neuroprotective and neurodegenerative properties and they have been tested for their potential effects in reversing pathological hallmarks of AD both *in vitro* and *in vivo* [56,57]. Trichostatin-a was found to be the top candidate repurposed drug in our list as it alters the expression of 66 out of 80 NRF2-related differentially expressed genes from our analysis. It has been indicated that the therapeutic effect of trichostatin-a on AD is associated with the KEAP1-NRF2 pathway [58]. Parthenolide is a biologically active sesquiterpene lactone and it has been reported to improve cognitive function and reduce levels of TNF- $\alpha$  and IL-6 in the cortical and hippocampal regions of rats. It also appears that this compound inhibits neuroinflammation in different neuropathologies [59]. It has also been reported that entinostat reverses hypoacetylation and has beneficial effects in AD animal studies [57].

The five repurposed drugs were further validated in cell culture. As expected from several studies, curcumin was an NRF2 activator [60] and could be considered as a positive control. Indeed, it has been reported that curcumin increases NRF2 protein levels by inhibiting its main repressor KEAP1 through electrophilic interactions with specific cysteines in this protein [61]. However, the potential use of entinostat,

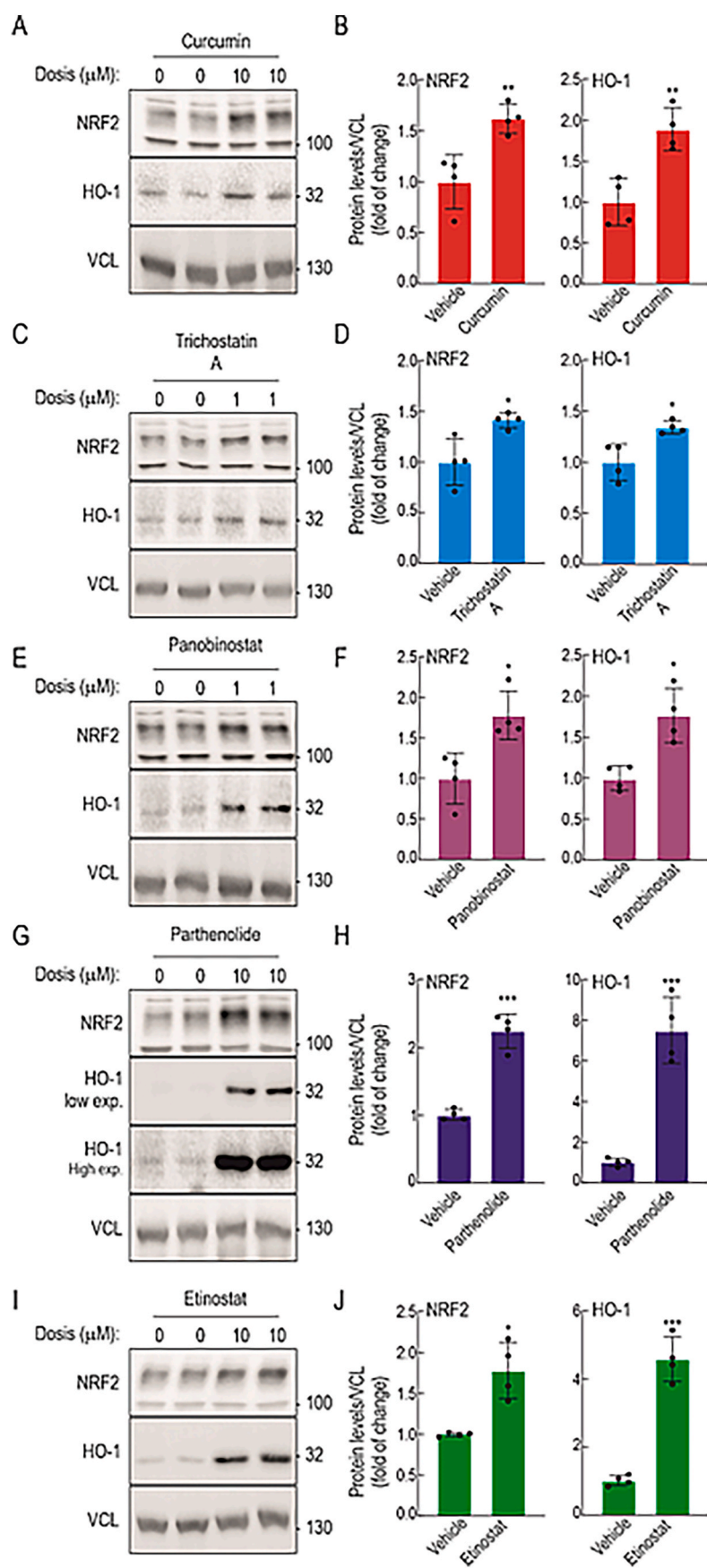
panobinostat, trichostatin-a, and parthenolide as NRF2 activators has not been consistently investigated. It was surprising to find the induction of NRF2 and the NRF2 signature by entinostat, panobinostat, trichostatin-a, because these three compounds are well-characterized histone deacetylase inhibitors and they might have reduced the expression of NRF2 coding gene, termed *NFE2L2*, in cancer cells. It is known that in Ewing sarcoma and osteosarcoma entinostat inhibits YB-1 deacetylation, decreasing *NFE2L2* expression and sensitizing cells to oxidative stress [62]. Most likely the different drug responses are due to variations in cell type, time of incubation, and drug concentration. For instance, in the case of trichostatin-a, a low dose activates the AKT/NRF2 pathway and upregulates expression of HO-1, SOD and CAT to stimulate the cellular defense mechanism against the oxidative stress induced by ionizing radiation [63]. Consistently, the drug concentrations that we used do not show evidence of toxicity (Fig. 6B). Probably the most interesting outcome of our study is the possible repurposing of the electrophilic compound parthenolide for neuroprotective therapy in AD. NRF2 activation by parthenolide has been superficially reported in the context of nephrotoxicity [64] adipocyte differentiation [65,66] and cancer resistance [67]. Notably, by increasing NRF2 protein levels, parthenolide increases the radiosensitivity of mouse xenograft tumors but protects normal prostate and bladder tissues against radiation-induced injury [68]. Mechanistically, in cancer cells this drug causes oxidation of thioredoxin, which in turn leads to KEAP1-mediated PGAM5 and Bcl-xL degradation and cell death. In contrast, in normal epithelial cells, parthenolide inhibits KEAP1, leading to increased NRF2 activity [68].

In summary, in our study we highlighted candidate repurposed drugs for AD using the differential expression of the *NFE2L2* and its partners. Through a four-step computational meta-analysis procedure we performed a validity check of our findings. Some of the candidate repurposed drugs are already in ongoing clinical trials for AD and some others have already shown their relevance. Finally, our *in silico* analysis, resulted in 5 candidate repurposed drugs that were further validated in cell culture. These five candidates demonstrated the ability to enhance NRF2 activity as indicated by the activation of a luciferase reporter. Furthermore, in TH22 cells derived from the hippocampus, they exhibited an increase in NRF2 protein levels and enhanced transcriptional signatures of NRF2. We expect that our proposed candidate repurposed drugs will serve as prime candidates for future AD research and clinical experiments.

#### Authors' contributions

Supervision of the study: G.M.S. Conception and design of the study:





**Fig. 7.** Curcumin, Trichostatin-a, Panobinostat, Parthenolide, and Etinostat induce NRF2 and its bonafide target HO-1. **A)** representative immunoblots of NRF2, HO-1 and VCL as a loading control subjected to 10  $\mu\text{M}$  Curcumin for 16 h. **B)** densitometric quantification of NRF2 and HO-1 protein levels from representative immunoblots from **A**, expressed as a ratio of VCL. Data are mean  $\pm$  S.D. ( $n = 4$ ). **C)** representative immunoblots of NRF2, HO-1 and VCL as a loading control subjected to 1  $\mu\text{M}$  Trichostatin-a for 16h. **D)** densitometric quantification of NRF2 and HO-1 protein levels from representative immunoblots from **C**, expressed as a ratio of VCL. Data are mean  $\pm$  S.D. ( $n = 4$ ). **E)** representative immunoblots of NRF2, HO-1 and VCL as a loading control subjected to 1  $\mu\text{M}$  Panobinostat for 16h. **F)** densitometric quantification of NRF2 and HO1 protein levels from representative immunoblots from **E**, expressed as a ratio of VCL. Data are mean  $\pm$  S.D. ( $n = 4$ ). **G)** representative immunoblots of NRF2, HO-1 and VCL as a loading control subjected to 10  $\mu\text{M}$  Parthenolide for 16h. **H)** densitometric quantification of NRF2 and HO1 protein levels from representative immunoblots from **G**, expressed as a ratio of VCL. Data are mean  $\pm$  S.D. ( $n = 4$ ). **I)** representative immunoblots of NRF2, HO-1 and VCL as a loading control subjected to 10  $\mu\text{M}$  Etinostat for 16h. **J)** densitometric quantification of NRF2 and HO-1 protein levels from representative immunoblots from **I**, expressed as a ratio of VCL. Data are mean  $\pm$  S.D. ( $n = 4$ ). \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  vs. vehicle according to Student's *t*-test.

M.M.B. and G.M.S. Collection, analysis and interpretation of data: M.M.B. and G.M.S. Cell Culture Experiments and analysis of the corresponding data: R.F.G. and A.C. Drafting the article/revising it critically: M.M.B., R.F.G., A.C. and G.M.S.

## Declaration of competing interest

None declared.

## Data availability

No data was used for the research described in the article.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.redox.2023.102881>.

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