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Building a pipeline to discover and validate novel therapeutic targets and lead compounds for Alzheimer's disease

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Abstract

Cognitive decline, Alzheimer's disease (AD) and other causes are major public health problems worldwide. With changing demographics, the number of persons with dementia will increase rapidly. The treatment and prevention of AD and other dementias, therefore, is an urgent unmet need. There have been considerable advances in understanding the biology of many age-related disorders that cause dementia. Gains in understanding AD have led to the development of ante-mortem biomarkers of traditional neuropathology and the conduct of several phase III interventions in the amyloid- β cascade early in the disease process. Many other intervention strategies are in various stages of development. However, efforts to date have met with limited success. A recent National Institute on Aging Research Summit led to a number of requests for applications. One was to establish multi-disciplinary teams of investigators who use systems biology approaches and stem cell technology to identify a new generation of AD targets. We were recently awarded one of three such grants to build a pipeline that integrates epidemiology, systems biology, and stem cell technology to discover and validate novel therapeutic targets and lead compounds for AD treatment and prevention. Here we describe the two cohorts that provide the data and biospecimens being exploited for our pipeline and describe the available unique datasets. Second, we present evidence in support of a chronic disease model of AD that informs our choice of phenotypes as the target outcome. Third, we provide an overview of our approach. Finally, we present the details of our planned drug discovery pipeline.

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Keywords

Alzheimer's disease; systems biology; targeted proteomics; RNAi; small molecule screen

1. Introduction

AD is the most common cause of dementia in the elderly. Its occurrence will increase markedly in the coming decades, making the prevention and treatment of cognitive impairment due to AD a major public health priority. Over the past 25 years, the aging and dementia research community has made progress in identifying risk factors for the disease and in characterizing the clinical and pathologic features of the disease [1-9]. Research findings led to a reconceptualization of AD as a chronic disease with dementia as its final stage, i.e., AD is associated with a long subclinical phase at which the characteristic pathology is present in the absence of overt cognitive impairment, followed by subtle cognitive deficits and then obvious mild cognitive impairment (MCI) due to AD, and ultimately to AD dementia with new clinical criteria [10-12]. Pathologic criteria have likewise been revised [13]. The new knowledge is being translated into clinical settings via the development of biofluid and neuroimaging biomarkers of the commonly accepted pathologies, which are now being incorporated into clinical trials of disease modifying pharmacotherapies [14-31].

The gains in our understanding of the risk factors and clinical and pathologic features of AD have not been translated into effective treatments to reduce the suffering associated with dementia due to AD, or to delay disease onset. Few interventions have been approved by the Food and Drug Administration (FDA) for the treatment of AD. The identification of the cholinergic deficit in AD in the 1980s led to the approval of four acetylcholinesterase (AChE) inhibitor treatments of dementia due to AD in the 1990s [32,33]. A decade later, an N-methyl D-aspartate (NMDA) receptor antagonist also was approved [32,33]. These treatments only have modest symptomatic effects for relatively short time periods. A number of disease modifying clinical trials have been conducted over the past decade, many focused on amyloid metabolism [34-45]. Despite the investment of billions of dollars by industry, NIH, and foundations, none of these trials met their combined endpoints of showing cognitive change and a meaningful difference in daily function. There have been very few primary prevention studies for AD and these too have failed [46]. The investments continue [47-48]. A general consensus in the field has emerged that interventions need to occur earlier in the disease process and that power can be improved by studying people at high risk. A number of studies are underway targeting amyloid with this approach [49-51]. However, another consensus is that other targets need to be investigated, and there are several other pharmacologic and non-pharmacologic interventions that are ongoing, planned, or under consideration [52-61]. Overall, despite remarkable progress, and billions of dollars of investment, the current symptomatic treatment regimens have small effects with limited clinical and public health impact, and there are no approved treatments to delay the onset of MCI due to AD or AD dementia. Thus, new approaches to the identification and validation of novel druggable targets are urgently needed.

The National Institute on Aging (NIA) held a two day open meeting (May 14-15, 2012): Alzheimer's Disease Research Summit 2012: Path to Treatment and Prevention with presentations and discussants from across the United States [62]. There were six sessions each accompanied by a set of recommendations at the conclusion of the meeting that came from a writing committee composed of a subset of speakers. NIA staff used the recommendations to develop a small slate of requests for applications released in January of 2013. One, *Interdisciplinary Approach to Identification and Validation of Novel Therapeutic Targets for Alzheimer's Disease* (RFA-AG-13-013), was to support "...integrative, interdisciplinary research focused on the identification and preclinical validation of novel therapeutic targets within molecular networks involved in different stages of Alzheimer's disease (AD) pathogenesis." We were recently awarded one of three such grants [63]. Here we describe the two cohorts that provide the data and biospecimens being exploited for our pipeline and describe the available unique datasets. Second, we present evidence in support of a chronic disease model of AD that informs our choice of phenotypes as the target outcomes. Third, we provide an overview of our approach. Finally, we present the details of our planned drug discovery pipeline. The challenge posed by the prevention and treatment of AD is daunting. We recognize that a variety of complementary approaches are needed and that failures will outnumber successes by a large number. Thus, we present this approach as one of many strategies that may ultimately lead to robust interventions that improve the health and well-being of our aging population.

2. Available Data and Biospecimens

The data and biospecimens come from two cohort studies of aging and dementia that include organ donation at death: the Religious Orders Study and Rush Memory and Aging Project [64,65]. The Religious Orders Study began data collection in 1994 and the Memory and Aging Project in 1997. In both cohort studies, older persons without known dementia sign an informed consent for annual clinical evaluation and donation of ante-mortem blood. They also sign an anatomic gift act for brain donation. The studies were conducted in accordance with the Declaration of Helsinki and were approved by the Institutional Review Board of Rush University Medical Center in Chicago. Both studies have a rolling admission, and nearly 3,000 persons have enrolled to date. The studies have a large common core of clinical and pathologic data that allow efficient merging of data for joint analyses.

2.1. Clinical data collection

The clinical evaluation has been previously described in detail [66-71]. Briefly, it includes 19 cognitive function tests and uses accepted and validated procedures to diagnose incident MCI and incident AD and other dementias. The follow-up rate exceeds 95% with more than 800 cases of incident MCI and more than 600 cases of incident dementia to date. Of the cognitive function tests, 17 can be merged and summarized as a global measure of cognition; subsets of tests are used to summarize measures of episodic, semantic, and working memory, perceptual speed and visuospatial ability. Numerous other age-related phenotypes are available in both cohorts [64,65].

2.2. Neuropathologic data collection

The autopsy rate exceeds 90% with more than 1,200 autopsies to date. As people enroll without dementia, there are autopsies from people representing the full range of cognition at death including a third without cognitive impairment, a quarter with MCI, and the rest with AD and other causes of dementia. Further, the studies began recruitment two decades ago such that participants now have up to 20 years of annual clinical evaluations prior to death. The detailed neuropathologic evaluation has been previously described [72-79]. Briefly, the neuropathologic assessment quantifies the most common pathologies that contribute to dementia. Pathologic indices of AD include accepted criteria for a pathologic diagnosis of AD. In addition, there is a measure of the burden of AD pathology based on counts of neuritic and diffuse plaques, and neurofibrillary tangles. The counts are standardized as a global measure of AD pathology. This is complemented by percent area occupied by amyloid- β by image analysis and neurofibrillary tangle density by stereological counting. An amyloid stain is also used to identify amyloid angiopathy. The age, location, and size of all macroscopic infarctions are recorded, and several brain regions are examined for microscopic infarctions. Lewy bodies are identified with antibodies to α -synuclein. Hippocampal sclerosis is identified on H&E. Finally, pathologically phosphorylated TAR DNA-binding protein 43 (TDP-43) cytoplasmic inclusions are also assessed.

2.3. Genome-wide multi-level omics datasets

Our AD target discovery pipeline will leverage the availability of a unique, genome-wide, multi-level omics dataset generated from frozen postmortem dorsolateral prefrontal cortex (DLPFC) brain tissue from about 1000 non-Hispanic, white study participants. The choice of DLPFC was made in 2008 when the creation of these datasets was first initiated. Several regions were considered and debated before settling on this region. While the focus of this pipeline is AD, the primary consideration was to identify a region involved in many disease processes and conditions under investigation in the parent cohort studies which extend well beyond AD, CVD, LBD and HS. They include parkinsonism and gait disturbance, motor neuron disease, physical frailty, and a wide variety of other phenotypes that are affected by aging or modify the effect of aging on adverse health outcomes, including sleep and circadian rhythms, pain, menopause, social cognition, experiential factors, depression, psychological traits, behavioral economics, decision making, literacy, nutrition odor identification, and well being [80-102]. Thus, we were looking for a single region that would be of high value for AD but would also represent an area of importance for the fullest range of phenotypes of interest to allow the omics data to be repurposed for the highest and best use. This led us to select the DLPFC which had been implicated in an extraordinarily wide range of these phenotypes [103-119].

2.3.1. Genotypes—DNA was extracted from whole blood, lymphocytes, or frozen brain tissue. Genotyping was done on the Affymetrix Genechip 6.0 or Illumina OmniQuad Express platform as described [120,121]. Datasets underwent the same quality control (QC) analysis using PLINK [122]. We applied standard quality metrics such as a genotype success rate >95% and a Hardy-Weinberg $p > 0.001$. EIGENSTRAT was used to identify and remove population outliers [123]. Genotype imputation was performed with BEAGLE (version:3.3.2) and generated dosage data on >35 million SNPs for each individual using the

1000 Genomes Project (2011 Phase 1b data freeze) as a reference. We limit our analyses to the ~7.5 million SNPs with an MAF ≥ 0.01 and imputation quality (INFO) score >0.3 . These data are currently available on more than 2,000 persons, including more than 1,000 autopsied participants.

2.3.2. DNA methylation—We generated DNA methylation profile data using the Illumina Infinium platform and the HumanMethylation450 beadset from frozen DLPFC [124-127]. This platform contains a total of 485,513 probes which covers 21,231 genes (17.2 probes per gene), and covers a total of 26,658 CpG islands (96% of those annotated so far) as well as extended flanking regions defined as “shelves” and “shores”. Data were processed using Genome Studio software Methylation Module v1.8. Poor quality probes (detection p-values >0.01) and samples with $<450,000$ good quality CpG probes or low quality bisulfite conversion rates were discarded. Missing beta-values were imputed and approximated using k-nearest neighbor algorithm with $k=100$. We used Principal Component Analysis and selected samples having principal component 1, 2 and 3 (PC1, PC2 and PC3) values within ± 3 standard deviations from the means. We then removed duplicate and mis-annotated samples. We removed probes which contain a SNP as well as probes that have extensive homology with the X chromosome. We only consider autosomes in our analyses. This resulted in more than 420,000 CpG sites being interrogated in nearly 750 persons.

2.3.3. Histone acetylation—Chip-seq was done using Broad's Illumina sequencing platform from frozen DLPFC to generate data using the H3K9 histone acetylation (H3K9Ac) mark that is associated with actively transcribed genes [128]. We used 36-base pair single-end reads aligned to the reference human genome using MAQ software [129]. Duplicated reads were flagged using “MarkDuplicates” module from Broad's Picard software pipeline (<http://picard.sourceforge.net/>). The quality scores for all the bases were recalibrated using “TableRecalibrator” module from GATK software [130]. We identified histone binding peaks with the MACS software [131]. We used “intersectBed” module from BEDTools to find similar or overlapping regions of histone modifications from different individuals [132]. Using the same module with different options, we found individual-specific regions of histone modifications over the genome. This resulted in a median of about 180,000 histone H3K9Ac peaks in nearly 750 subjects.

2.3.4. Micro RNA—We collected expression profiles for nearly 700 miRNAs from frozen DLPFC using the NanoString nCounter miRNA expression assay [133]. We pre-processed the dataset to retain miRNAs with a call rate $>95\%$ and an absolute value ≥ 15 in at least 50% of the samples. The dataset was normalized using variant stabilization normalization. The batch effects were corrected using Combat specifying the cartridges as batches. In all, more than 300 miRNAs meet these pre-processing and expression criteria in more than 700 subjects.

2.3.5. Next generation RNA-sequencing transcriptome—The same aliquot of DLPFC RNA used for the miRNA experiment was used to generate cDNA libraries by the Broad's Genomics Platform using the strand specific dUTP method with poly-A selection [134-136]. We required RNA integrity (RIN) score >5 and a quantity threshold ($>5\mu\text{g}$) for

each sample. Sequencing was performed on the Illumina HiSeq with 101bp paired-end reads and achieved coverage of 150M reads of the first 12 samples which serve as a deep coverage reference (2 males and 2 females each unimpaired, MCI, and AD dementia). The remaining samples were sequenced with coverage of 75M reads. The libraries were constructed and pooled according to the RIN scores. This resulted in measures for nearly 80,000 genes and isoforms from nearly 550 persons. A second batch of more than 450 samples is currently being processed.

3. The Chronic Disease Model of AD

Over the past quarter century, data from a variety of sources has converged to support a chronic disease model of AD that recently led to revised clinical and pathologic criteria [10-12,137]. Support for this model came in part from data from the two cohort studies that provide data and biospecimens for our pipeline. The clinical and pathologic data are suitable for interrogation by a variety of approaches. Logistic and linear regression is used to examine cross-sectional associations with dichotomous and continuous measures. We also incorporate pathologic indices into mixed models to examine change in cognition as the dependent variable; these can be linear mixed models or change point models [64, 138-145]. Pathologic indices and cognitive change slopes derived from linear mixed models can also be incorporated into mediation, structural equation, and latent variable models [146-148].

3.1. AD dementia and MCI have a long preclinical phase

By definition, a clinical diagnosis of dementia due to AD requires a history of cognitive decline [10]. Data from our study and others demonstrate that the trajectory of cognitive decline begins several years prior to when the diagnosis of AD is made [71,149-154]. We illustrate this in Fig 1. Figure 1a illustrates the raw data trajectories for a random sample of participants who never developed AD (light green) and those who were diagnosed with clinical dementia during the study follow ups (pink), superimposed by the model predicted mean trajectories. For participants who developed incident dementia, we introduced an inflection point at the time of clinical diagnosis to allow for additional change in the rate of cognitive decline. Considering that the timing of clinical diagnoses can be imprecise, we also developed random change point models to estimate empirically the onset of acceleration in the rates of change. These data suggest that global cognitive function begins to decline several years prior to the time a clinical diagnosis of AD dementia is made.

Similarly, Fig 1b illustrates the raw data trajectories for a random sample of participants who never developed cognitive impairment (light blue) and those who were diagnosed with clinical MCI (gray), superimposed by corresponding model predicted mean trajectories. As in Figure 1a, we allow for additional change in the rate of cognitive decline at time of clinical diagnosis. These data suggest that global cognitive function begins to decline several years prior to the time a clinical diagnosis of MCI due to AD is made.

3.2. The relation of AD pathology to cognition is a continuum

Data from our group and others demonstrate that AD pathology is present in persons with MCI and also in persons without dementia or MCI [73,76, 155-159]. In fact, as with change

in cognition before and after AD, the relation of AD pathology to cognition is similar across the spectrum from normality to MCI to AD dementia. We illustrate this in Fig 1. The scatter plot illustrates the relation of the global measure of AD pathology to level of global cognition proximate to death (Fig 1c). The light blue dots represent persons without dementia or MCI, the light green dots persons with MCI, and the pink dots persons with AD dementia. The solid lines are the model derived regression lines controlling for age, sex and education. The blue, green, and red lines represent the regression lines for average participants (i.e. female at mean age and mean level of education) among those without dementia or MCI, MCI, and AD dementia respectfully.

We demonstrate a similar continuum in the relation of AD pathology to change in cognition over time (Fig 1). We first used a linear mixed model to develop person-specific slopes. The scatter plot illustrates the relation of the global measure of AD pathology to the slope of change in cognition over multiple years prior to death (Fig 1d). The light blue dots represent persons who died without dementia or MCI, the light green dots persons who died with MCI, and the pink dots persons who died with AD dementia. The solid lines represent model derived regression lines for average participants (i.e. female at mean age and mean level of education) illustrating the rather continuous relationship between the burden of AD pathology at autopsy and the rate of change in global cognition over the years prior to death.

3.3. Mixed pathologies and AD dementia

It is now generally accepted that both the clinical and pathologic manifestations of AD are on a continuum from normality, to MCI to AD dementia [73,76,160-165]. In other words, AD starts with a pathophysiologic process that is silent at first, followed by subtle cognitive impairment, MCI due to AD and finally AD dementia. AD is also a disease of aging and coexists with several other common age-related pathologies that impair cognition including cerebrovascular disease (CVD), Lewy bodies (LB), hippocampal sclerosis (HS), and TDP-43. The result is that mixed disease is not only the most common cause of dementia, but also the most common cause of the clinical phenotype of AD dementia [73]. Finally, it should be noted that the existing pathologies only account for about half the variability in person-specific slopes of cognitive decline [100,101]. Thus, other pathologies associated with cognitive decline and dementia await discovery. For example, TDP-43 was originally thought to be a relatively rare pathology associated with Amyotrophic Lateral Sclerosis (ALS) and Frontotemporal Lobar Degeneration (FTLD) [166]. However, recent data suggests that it is frequently found in older persons with and without dementia [167,168]. Further, we recently showed that phosphorylated TDP-43 cytoplasmic inclusions were associated with cognitive decline, controlling for AD, CVD, LB and HS [79]. Some of these “to be identified” pathologies will likewise contribute to the AD dementia phenotype.

3.4. Leveraging quantitative endophenotypes for drug target discovery

The chronic disease model of AD with mixed pathologies contributing to the AD phenotype raises a number of important limitations in the use of case-control designs for AD drug target discovery. First, while the vast majority of persons with AD dementia have pathologic AD, a substantial number of persons in the reference group have AD pathology. This can substantially reduce power in association studies. Second, since other known and unknown

pathologies contribute to the AD phenotype, genomic and other risk factors for co-existing pathologies (or no pathology) can emerge as risk factors for “AD” in association studies [139,161-171]. For example, in our studies, diabetes is associated with incident AD dementia [169]. However, at autopsy it is associated with macroscopic infarctions but not measures of AD pathology [170]. Thus, the phenotype can bias association studies in a way that cannot be overcome by sample size. To a large extent we are interested in all risk factors for dementia regardless of the specific biologic process, so the data that emerge from case-control studies are of fundamental value. However, if we need to be cautious when including them in our biologic architecture of AD as we can bias our conceptual model which can eventually lead to model failure with adverse effects on drug discovery efforts. Finally, since AD pathology only explains about a third of the variance of cognitive decline [140,141], we may overestimate the potential benefits of manipulating AD pathology in clinical trials leading to large but underpowered intervention studies.

We find strong data supporting the use of endophenotypes for identifying risk factors in general, including genomic variants [172]. Thus, we advocate using a quantitative measure of AD pathology complemented by the trajectory of cognitive decline as the phenotypes of interest for AD drug target discovery [128,173]. We and others have successfully harnessed quantitative endophenotypes to identify genomic variants related to both AD pathology and cognitive decline in both discovery and validation efforts [142-146,174-187]. In fact, one of the first uses of neuropathology as an endophenotype was the association of *APOE* with cortical deposition of amyloid- β [188].

While we *a priori* selected the complementary phenotypic outcomes of a quantitative measure of AD pathology and the trajectory of cognitive decline, we recognize that this is but a first step. The pathophysiological processes of interest are likely quite complex such that two or more distinct etiologies may result in both AD pathology and cognitive decline yet require different, specific interventions. This is certainly the case with CVD, for example, which may require pharmacologic interventions for hypertension, diabetes, and high cholesterol, in addition to diet and other lifestyle changes. Hypertension alone is a complex condition frequently requiring a combination of two or three different medications. We can already envision a similar set of complex processes for amyloid deposition that might require interventions targeting β - and/or γ -secretase, in addition to strategies to enhance amyloid- β clearance. We believe our dataset is ideally suited to meet the range of challenges. As more clues emerge regarding the complexity of AD, we can incorporate the data into our modeling strategy. For example, we may enrich the modeling with genomic variation in known AD risk genes, or we may enrich for residual cognitive decline, i.e., the residual cognitive trajectory that cannot be accounted for by known dementia pathologies [140-141].

4. Overall Approach

The overall goal of the drug discovery pipeline is to discover and validate novel therapeutic targets and lead compounds for AD. It brings together a strong and unique multi-disciplinary team of epidemiologists, geneticists, neurologists, neuropathologists, biostatisticians, cellular neuroscientists, computational biologists, medicinal and bioanalytical chemists, and

clinical trialists. The outcome of interest is a quantitative measure of AD neuropathology. This is complemented by repeated measures of cognition over up to two decades on about 1000 prospectively-followed, community-dwelling, older adults initially free of dementia all of whom were organ donors and came to autopsy. . It uses a systems biology approach to integrate genome-wide and other multi-level “-omics” datasets including DNA methylation, histone acetylation (H3K9Ac), miRNA, and mRNA transcriptome generated from frozen human dorsolateral prefrontal cortex tissue (DLPFC), a key node in the neural network that subserves cognition, and is vital to the execution of a wide range of other aging phenotypes. It integrates quantitative measures of AD and other common pathologies including Lewy bodies, TDP-43, hippocampal sclerosis, and macro- and micro-infarctions into the analyses to identify genes in networks attributable to AD and other common neuropathologies in the sequence of molecular events leading from health to cognitive decline. The approach balances the identification of novel targets within known molecular pathways, e.g., targets leading to AD pathology, with the identification of targets in novel networks; it balances the identification of targets in pathways with therapeutics that have previously been the subject of phase I studies that can be repurposed with the need to identify the most robust targets without regard to repurposing. Finally, the approach balances the need for targets in pathways that drive pathology and the development of AD for dementia prevention, while not losing sight of the many individuals who suffer from the disease and could benefit from more robust symptomatic treatments.

4.1. Target characteristics for an ideal compound

- Target is a nodal point in a network related to AD pathologic and clinical quantitative phenotypes identified by integrative analyses of genomic, epigenomic, and transcriptomic data from human brain tissue
- Target is expressed and translated in the human brain and related to AD pathologic and clinical phenotypes
- Target, a nodal point in the AD network, is confirmed with *in vitro* RNA interference (RNAi) screen in human neurons or astrocytes derived from human induced pluripotent stem cells (iPSC)
- Target's druggability is demonstrated by small molecule screen of human neurons or astrocytes derived from human iPSC
- Target could rapidly be moved into human clinical trials
 - Target is druggable by a known, FDA approved drug to minimize need for new safety data
- Target is druggable by an unapproved drug that has been studied in Phase I trials and found to be safe to bypass preclinical drug development
- Target would be in a pathway linking amyloid to tangles to leverage amyloid and more recent tau imaging technologies

5. Drug Discovery Pipeline

Our drug discovery pipeline will provide lead compounds that disrupt the molecular networks that lead to the accumulation of AD neuropathology and trigger the neurodegenerative process that leads to cognitive decline, and ultimately to the clinical manifestations of cognitive impairment and dementia due to AD. The pipeline has four components outlined in Figure 2.

5.1. Systems biology component

The first component employs a systems biology approach to integrate genome-wide genotype data, with (a) DNA methylation, H3K9Ac acetylation, miRNA, and mRNA transcriptomic data from frozen human DLPFC, a brain region at the hub of neural networks subserving cognition, and (b) AD pathologic and clinical quantitative traits, to nominate genes, and therefore proteins, from networks and nodes involved in the molecular pathways leading to AD and perhaps other dementias. Systems biology methods for integrating multi-level “omics” data are evolving rapidly. While there have been network analyses in AD transcriptomic data [189], there is currently no single method to derive molecular networks that relate to a clinical condition. We therefore discuss several options that exist today to illustrate our general approach. We leverage the fact that the different genomic features (e.g., genetic variation, CpG, H3K9Ac peak, miRNA) all ultimately influence mRNA expression, an essential step leading to protein translation. We will therefore use the RNA-Seq data as the target on which the effects of the different genomic features converge. This is illustrated in Figure 3. We will deploy new methods to model our RNA data that identify regulators within molecular networks, such as Module Networks, LirNet, or Elastic Net regression that performs regression and variable selection simultaneously. These network analyses generate a hierarchical structure of effects that order the sequence of events to which the different genomic features contribute. In short, we will identify which combination of genetic variants, CpGs, H3K9Ac peaks, and miRNAs associated with AD endophenotypes control the expression of a given set of co-expressed genes. Then, we will integrate results across gene sets to assess whether there are interactions between the effects of different molecular networks.

These analyses will be complemented by other approaches such as (1) DAPPLE that leverages reference protein:protein interaction maps to assess whether associated genes physically interact, and (2) evidence for natural selection that can link the evolutionary history of different genes. We recently used these strategies to successfully link several AD susceptibility genes into a functionally coherent unit [190]. Thus, we will not rely on any single method but will employ a suite of complementary methods and will integrate results to best assemble the functional consequences of genetic variants and the various genomic features within the susceptibility loci that have independent effects on the AD endophenotypes. Based on available resources, we will identify a subset of ~300 targets (genes) from these molecular networks to move forward in our experimental pipeline. The targeted genes will cover a range of potential high value targets and will also include protein targets that are, *a priori*, more tractable and may therefore be more rapidly deployed into human trials.

5.2. Targeted proteomics component

The second component of the pipeline will use targeted liquid chromatography-selected reaction monitoring/mass spectrometry (LC-SRM/MS) proteomics to ensure that the targeted genes are expressed and translated in human brain [191,192]. LC-SRM/MS exploits the capabilities of triple quadrupole (QQQ) mass spectrometers to provide significant improvements in sensitivity, dynamic range, and coefficient of variation for quantitative proteomic analysis compared to other approaches [193]. The LC-SRM/MS approach measures pre-selected analyte ions in two stages of mass selection resulting in a set of chromatographic traces with the retention time and signal intensity for a specific transition. Modern QQQ mass spectrometers can monitor a few hundred protein targets in a single analysis making SRM/MS attractive for high throughput projects. Combination of the elution time and two levels of mass selection result in high specificity [194-196]. We currently process the data with SkyLine software and express the relative abundances of the quantified peptides (i.e., measured protein variants). These data can be analyzed with standard regression techniques, pathway analyses, and structural equation models to provide empirical support for biologic pathways linking proteins to AD endophenotypes [146]. The empirical data will be fed back to the network modeling stage to refine the network analyses. This component ensures that the candidate genes generated from the systems biology component above are translated in the human brain and that the measured protein variants themselves are related to AD endophenotypes.

5.3. Functional validation component

The third component will use RNAi screens to knock down and overexpress each of the selected genes in neurons or astrocytes differentiated from human iPSCs. We currently plan to interrogate 7 different constructs including an average of 6 shRNA and 1 Open Reading Frame (ORF) in each cell type. Perturbing the function of the cell using multiple independent constructs for each gene will ensure a robust assessment of its function. We currently plan to employ the 3' end RNA sequencing (RNAseq) method to generate a digital gene expression (DGE) measure for each target. This will provide an efficient, high throughput approach to generating the data required for the analysis of transcriptional networks. RNA profiles derived from each experimental condition will allow us to empirically reconstruct the molecular networks in the target, human cell types to confirm the pathways identified in our initial integrative analyses: we will also be able to address the directionality of relationships in these networks, enriching the information that we will use to identify the key nodes in the network [197,198]. This component of our pipeline has several purposes: (1) it will refine the networks and confirm that the genes nominated in systems biology analyses actually have the expected effects when they are disrupted on an individual basis; (2) it will identify other genes which may be involved in the network because they have similar functional consequences when their expression is perturbed; (3) it will identify transcriptional programs or "gene sets" that, *in vitro*, capture aspects of the function of a given pathway and can be used as outcome measures in future drug screening; and (4) it will also identify nodal points in each network, "hubs" for a given pathway that may make particularly effective targets for the disruption of a given cellular pathway. Overall, these experiments will also offer insights into the mechanism and cell-autonomous

effects of the target pathways that are originally determined using cortical tissue-derived data.

5.4. Small molecule screen component

Having (1) confirmed that these proteins are expressed in the aging brain and that they are associated with pathologic and/or clinical AD quantitative traits, and (2) functionally validated the brain-derived networks using the iPSC-derived neurons and astrocytes to identify the nodal genes that play a central role in each network, we will select up to three molecular targets to be interrogated by high throughput small molecule screening of the same iPSC-derived neurons and astrocytes that were used in our RNAi screening paradigm. These target cells, while introducing additional complexity into the screening paradigm, offer the unique advantage of being the best approximation of the target cells for AD therapy. An important challenge of our experimental pipeline is that, by design, we are driven by empiric observations from the first three stages that culminate in the selection of target nodal points for small molecule screening; thus, we do not know, *a priori*, which targets or even which molecular pathway will be the focus of our small molecule screens in this stage of the pipeline. Nonetheless, we must select and optimize the experimental assays that will be deployed in a high-throughput manner; while such efforts are critical, the large repertoire of existing assays using transcriptional profiling, metabolic measures, or the measurement of different types of analytes ensures that an assay can be designed to interrogate the vast majority of possible target proteins that are nodal points in the AD networks. A more challenging issue is that, while the targeting of certain classes of proteins such as tyrosine kinases is well documented and has been performed extensively in different experimental paradigms, the best target proteins that emerge from the final, validated networks may be in molecular classes that are traditionally thought of as being difficult to target. To address the issue, we included the Broad Institute's Therapeutics Platform as an integral member of our team of investigators. In addition to providing experience with a large variety of experimental paradigms, the platform has a focus in targeting cellular processes that are classically thought to be difficult to manipulate with small molecules. To this effect, it has implemented a Diversity Oriented Synthesis (DOS) strategy [199] in generating its library of more than 100,000 compounds. DOS is designed to facilitate all chemistry-based stages of drug discovery – (i) providing rich structural diversity similar to natural products, (ii) full stereochemical structure activity relationships (SSAR) from the primary screen; (iii) allowing rapid medicinal and process chemistry; (iv) permitting the addition of functional handles to allow faster and more efficient target identification from phenotypic assays. Thus, the screening of our assays with this large library containing many novel compounds will open many avenues for rapid drug development and optimization.

6. Future Directions

We designed a comprehensive drug discovery pipeline for the identification of novel molecules that target our endophenotypes of interest, pathologic and clinical AD quantitative traits, and other neuropathologies of aging. It is grounded in a deep empirical characterization of the human neocortex in a representative sample of the older population and then experimentally validated prior to the development of experimental paradigms in the

target human cell types that are compatible with high-throughput compound screening. This experimental pipeline is modular, allowing the inclusion of additional validation strategies as needed, and flexible: it can be repurposed for the study of other central nervous system phenotypes, of which a wide range exist in the parent cohort studies. Thus, there are several directions which can be pursued in the future.

First, with the availability of the different neuropathologic outcomes in the two cohort studies, we are in an excellent position to potentially identify lead compounds that target different elements of the causal chain of molecular events leading to AD: the identification of targets involved not just in early amyloid pathology but also the accumulation of neurofibrillary tangles, for example. Data on cognitive decline and other cognitive phenotypes will also allow us to potentially treat patient populations that are at different locations along the trajectory towards AD, both in the asymptomatic and perhaps the early symptomatic phases of the disease. Further, the targeting of neurodegenerative disease pathways that multiple pathologies have in common would be of particular interest and could allow for more efficient clinical trial design. There is also the possibility of exploring potential synergies of individual compounds by combinatorial treatment aimed at discrete processes that independently contribute to the pathophysiological cascade of AD.

Second, while the lead compounds pursue their pre-clinical and clinical development, they will also provide excellent opportunities for experimental work as tool compounds. Such tool compounds can disrupt and further refine our molecular networks to elaborate our understanding of the sequence of events that lead most of us to begin a long process of accumulating AD neuropathology in middle age.

Third, the two cohort studies have a wide range of other phenotypes related to health and well being in the elderly. The DLPFC was explicitly chosen to be of relevance to many of the available phenotypes. Thus, our pipeline is scalable and can be leveraged to discover and validate novel therapeutic targets and lead compounds for a range of other pathologic and clinical phenotypes.

Fourth, an exciting element of AD whose role in AD susceptibility is emerging more clearly from genetic studies is the role of immune cells [189,200-202]. These cells – including resident microglia, infiltrating macrophages and other immunocytes - are present in the cortical tissue that we have profiled, and it is quite likely that some of the networks implicated in the cascade of AD pathophysiology that emerge from our integrative analyses will relate to the immune system. Using the same RNAi paradigm described above for iPSC-derived cells, these immunocyte networks will be dissected and reconstructed in primary human immunocytes to inform the selection of targets and the development of assays for deployment in a high-throughput screening. Inclusion of this element into our drug discovery strategy will provide a more complete model of three representative cell types involved in the accumulation of AD pathology: astrocytes, microglia/macrophages, and neurons.

7. Beyond AD

The current pipeline is focused on AD target discovery and validation. However, as noted above, measures of AD pathology only account for about a third of the variability of cognitive decline [140,141]. This results, in part, from the likelihood that the lesions themselves are not the direct cause of cognitive decline but are markers of the disease process. Ultimately, loss of cognition must result from the dysfunction and degeneration of the neural elements that subserve cognition, i.e., neurons, synapses, dendrites, dendritic spines. Some evidence suggests the amyloid- β oligomers may be causative of synaptic dysfunction, and there is some evidence of this in humans [203-205]. There is less human evidence of a contribution from soluble tau, but this may stem in part from technical difficulties [206]. We also discussed other known, including LB, TDP-43, hippocampal sclerosis, and CVD, in addition to mixed dementias. Together, with AD, the known pathologies still account for less than half of the variability of decline [140,141]. Again, there is evidence that soluble α -synuclein contributes to cognitive impairment [207]. However, residual decline remains. There are likely a number of additional non-specific additional contributors to neural degeneration that may or may not be associated with the known pathologies. The most discussed are oxidative stress and inflammation, but more recently autophagy and other factors have been proposed [208-210]. We are in the unique position of being able to model the person-specific slopes of residual cognitive decline, i.e., the cognitive decline that remains after accounting for known pathologies. In the future, we will be able to leverage that phenotype for target discovery to uncover additional nodes and pathways that may be obscured by known pathologies.

8. Conclusions

We outline a drug discovery strategy that is grounded in empirical data generated from the target organ (brain) of about one thousand individuals that are representative of our aging population and have been characterized in detail both ante-mortem and post-mortem. This unique approach avoids biases introduced by the study of small numbers of subjects in specialized clinical centers and by the lack of detailed phenotypic data prior to death. The integration of multiple different outcome measures (pathologic and clinical intermediate phenotypes) pertinent to different stages of the trajectory from normality to MCI due to AD to AD dementia also provides a diversity of targets that can ultimately be used to target treatments to individuals in the appropriate component of the trajectory to AD, i.e., primary, secondary, and tertiary prevention. This may ultimately offer a range of treatments that can be deployed sequentially or offered in combination to best target the state of an individual's aging brain: we may be able to tune our treatment strategies. A second, critical component of our strategy is the validation and refinement of the network models that we determine from our primary data: we confirm both (1) that the selected protein are actually expressed in the target tissue and are related to the phenotypes of interest and (2) that the relationships determined in our systems analysis are not artifacts of over-fitting complex data from a single set of subjects. As rich and large as our dataset is, we are cognizant of the intrinsic limitations of data modeling: our model will be an excellent platform for pathway selection, but our validation in the target tissue and the target human cell types present important components that will increase the likelihood of discovering meaningful compounds. The

drug screening process itself presents substantial advantages because it leverages the advantages of the DOS library and screens for compounds in a model system based on human cells of the target organ. This compelling drug development strategy is ambitious, but its risks have been carefully considered and mitigated at each step of the pipeline. The deployment of our strategy has now begun. While the identification of lead compounds is several years away, each step of the pipeline generates useful products for the scientific community (such as map of molecular networks from the integrated analyses) along the way, and each of these milestones will therefore illustrate our progress towards producing novel options for the therapy of AD. Success in identifying lead compounds will impact an important disease for our aging population, and it will also validate a drug discovery pipeline that can be repurposed for other neurologic and psychiatric diseases that affects us throughout the life course. Further, with a focus on drugs approved by the FDA that can be repurposed or that have undergone Phase I trials and are known to be safe [211], our strategy may enable us to bypass preclinical drug development for rapid deployment into human trials in some cases.

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Figure 1. Relations between AD phenotypes of cognitive decline, clinical diagnosis and AD pathology

Figure 1A [upper left]: Cognitive decline in participants with and without clinical AD dementia diagnosis. Light green lines are repeated raw cognitive scores for up to 15 years from 50 randomly selected participants who never received a diagnosis of AD dementia. The light pink lines are repeated raw cognitive scores for up to 15 years from 50 randomly selected participants who received a diagnosis of incident AD dementia. The dark green line is the linear mixed model derived mean trajectory for an average participant (i.e. female at mean age and mean level of education) who never received a diagnosis of AD dementia, and the red line is the change-point mixed model derived mean trajectory for an average participant who developed AD dementia. The inflection point was fixed at the time of clinical diagnosis of AD dementia. The figure illustrates that cognitive decline is detectable over multiple years prior to the AD diagnosis and the increase in the rate of decline following diagnosis. It also nicely illustrates the person-specific differences in rates of cognitive decline among persons who did and did not develop AD dementia.

Figure 1B [upper right]: Similar to Figure 1A but for incident MCI. Light blue lines are the repeated raw cognitive scores for up to 15 years from 50 randomly selected participants who never received a diagnosis of MCI. The gray lines are raw cognitive scores for up to 15 years from 50 randomly selected participants who received a diagnosis of incident MCI. The

dark blue line is the linear mixed model derived mean trajectory for an average participant (i.e. female at mean age and mean level of education) who never developed MCI, and the black line is the change-point mixed model derived mean trajectory for an average participant who developed incident MCI. The inflection point was fixed at the time of first MCI diagnosis. The figure illustrates that cognitive decline is detectable over multiple years prior to the MCI diagnosis and that there is an increase in the rate of decline following diagnosis. It also nicely illustrates the person-specific differences in rates of cognitive decline among persons who did and did not develop MCI.

Figure 1C [lower left]: Continuum in the relation of AD pathology to cognition proximate to death. On the horizontal axis are quantitative measures of AD pathology. On the vertical axis are scores of global cognitive function proximate to death. Light blue dots represent participants without dementia or MCI at death. The light green dots are participants with MCI at death, and the pink dots represent participants with AD dementia at death. The blue, green, and red lines represent the linear regression lines for average participants (i.e. female at mean age and mean level of education) among those without dementia or MCI, MCI, and AD dementia respectfully, adjusted for age, sex, and education. The figure illustrates the point that the relation between cognition and pathology is only slightly steeper in those with AD dementia. It also nicely illustrates the relatively poor correspondence between the burden of the classic pathologic indices of AD and the level of cognition proximate to death. Figure 1D [lower right]: Continuum in the relation of AD pathology to change in cognition over time. On the horizontal axis are quantitative measures of AD pathology. On the vertical axis are model derived estimates for personal specific slope of decline in cognition, from a mixed model controlling for age, sex, and education. Light blue dots represent participants without dementia or MCI at death. The light green dots are participants with MCI at death, and the pink dots represent participants with AD dementia death. The blue, green, and red lines represent the linear regression lines for average participants (i.e. female at mean age and mean level of education) among those without dementia or MCI, MCI, and AD dementia respectfully. The figure illustrates the relation between the rate of cognitive decline and pathology, which is only slightly steeper in those with AD dementia. It also nicely illustrates the relatively poor correspondence between the burden of the classic pathologic indices of AD and rate of cognition decline over multiple years prior to death.

Outline of the AD drug discovery pipeline

Figure 2. Outline of the AD drug discovery pipeline

Component 1: System biology component. This component employs systems biology to integrate multiple types of "omics" data [upper middle] generated from frozen human DLPFC [upper left] to identify AD molecular networks and nodes, and nominate > 300 genes, and therefore proteins, to move to components 2 and 3. A representative result of a network analysis [upper right] that uncovered a molecular pathway linking known AD susceptibility genes with AD pathology is shown in the upper right panel.

Component 2: Targeted proteomics component. This component uses LC-SRM/MS to quantify > 300 proteins from frozen human DLPFC from the same subjects used in component 1 to ensure that the targeted protein is translated and that its level is related our AD endophenotypes. A cartoon of the MS intensity as a function of LC elution time results in detection of different proteoforms (protein fragments) and can be used to generate relative protein abundance with high specificity and fidelity [middle left].

Component 3: Functional validation component [middle right]. This component uses RNAi to knock down and overexpress each of the genes identified in component 1 in iPSC-derived neurons and/or astrocytes to confirm the identified pathways, address the directionality of relationships in these networks, and enrich the information that we use to identify the key

network nodes. The picture shows iPSC-derived neurons *in vitro* (stained for neuronal marker TUJ1 in green).

Component 4: Small molecule screen component [bottom]. This component will use high throughput small molecular screening of the same iPSC-derived neurons and astrocytes used in component 3 to identify compounds affecting the selected target genes. It will use the Broad Institute's Therapeutics Platform.

Figure 3. Illustration of the systems biology component

Our approach leverages different genomic features (e.g., genetic variation, CpG, H3K9Ac peak, miRNA) to be integrated with mRNA expression and our AD endophenotypes. Notably, all of the data are from frozen human DLPFC of the same participants [far left]. A representative genome-wide association SNP scan [top] that was used to identify novel variants associated with AD pathology such as SNPs in the amyloid precursor protein (*APP*) locus. Circos plot [upper middle] presenting the results of a genome-wide DNA methylation data with mRNA confirmation data to identify convergence of associations across multiple genomic layers to identify novel CpG dinucleotides at which the level of methylation is associated with AD pathology. Analysis of miRNA data [lower middle] reveals a network of up-regulated and down-regulated miRNAs in relation to four different AD pathologic traits. Representative tracks of ChIP-seq data [bottom] in the clusterin locus that illustrate the ongoing H3K9 chip-seq scan being used to identify novel acetylation peaks associated with AD pathology. Results from the different analyses are integrated using mRNA expression [right]: the panel shows the integration of the effects of two types of genomic features (DNA methylation and miRNA) that are identified as regulators of mRNA expression. These regulators are circled in the network diagram. The heatmap presents subjects in columns and genes in rows: genes are either up-regulated or down-regulated in a given subject.