Phenotypic Impact of Genetic Risk Pathways for Alzheimer's Disease

by

Daniel Felsky

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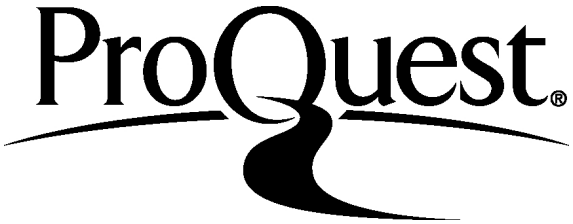

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Daniel Felsky

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Abstract

The contribution of genetic variation to risk for late-onset Alzheimer's disease is well-accepted; however, the roles of specific mutations within established risk genes are not clear. Comprehensive datasets with informative *in vivo* and postmortem biomarkers now offer the opportunity to understand when, where, and how mutations within these genes individually exert their effects on the brain. Moreover, it is known that many of these genes interact at the pathway level, and therefore genetic effects should also be considered in context using gene-gene interaction approaches. I hypothesized that common functional variants modifying established Alzheimer's risk pathways would demonstrate a) independent effects and b) synergistic effects on human brain structure and other Alzheimer's biomarkers. First, the Apolipoprotein E (*APOE*) gene ε4 allele was found to be associated with white matter integrity in an age-dependent manner. Second, mutations within the sortilin-like receptor (*SORL1*) gene were associated with differences in white matter integrity, *SORL1* gene mRNA expression, and amyloid

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neuropathology that suggested an early genetic risk mechanism beginning as early as childhood. Third, a translocator protein (*TSPO*) gene variant known to alter TSPO binding characteristics was found to have no direct effects on inflammatory and cerebrovascular brain changes in over 2 300 elderly subjects. Finally, based on evidence from recent human stem cell experiments, RNA sequencing was used to identify a novel interaction of gene variants across the *SORL1* gene with the brain derived neurotrophic factor (*BDNF*) Val66Met polymorphism regulating isoform-specific *SORL1* expression related to amyloid pathology and brain structural alterations. Altogether, these experiments demonstrate that some genetic modifiers of AD risk pathways are linked either directly via biochemical function or indirectly via the convergence of pathways they influence. These studies have begun to parse the immense heterogeneity of the Alzheimer's disease diagnosis as well as uncover distinct genetically-defined molecular subtypes of at-risk individuals who should be targeted in future therapeutic trials. Novel interventions designed to engage specific neural circuits or molecular pathways would be of most benefit to the molecular subtypes in which they are most greatly altered.

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Acknowledgments

I am endlessly grateful for the people and opportunities that have shaped my experience over the last several years. I am among a very lucky few to have been supervised by such exceptional mentors, Aristotle Voineskos and James Kennedy, without whom none of this would have happened. To the members of my Program Advisory Committee, Jo Knight, Jason Lerch, and Bruce Pollock, I cannot thank you enough for your support and stamina. You have all set examples that I strive to follow.

For the efforts and intelligence of my colleagues, by which I have been inspired over the years, I am also grateful: to Jon, my moral Sherpa, for his guidance, support, example of character, and editing assistance; to Tina B, for being a model of resilience, achievement, and friendship; to Tris and Colin, for their scientific passion and fervent debates; to Julie, for her optimism, dependable virtue, and flailing gestures; to Arash and Tina R, for their unparalleled intellect, enthusiasm, and humility; to Anne, for her uplifting drive and kindness; to Joe, for his command of comprehension, thirst for literature, and taste in music; to Nikhil, for his genuine scientific intrigue and companionship; to David, for his acerbic wit and commitment to the task; to Mallar, for his forthright and collaborative manner; to Vince and Nick, for their delightfully calm demeanors and remarkable insight; to Clement and Arun, for their patience and unwavering support of all in the Kennedy lab; and to Sajid, for the Turkish pizza and for starting it all.

It seems that academic training generates much suffering, to a degree greater than equally time-consuming pursuits. Some explain this as the discouraging endlessness of the quest for knowledge, but I think that well before that quest begins we struggle most with our self-perceptions of competence; the so-called imposter phenomenon. Like many graduate students, I often question my own abilities and in many ways this motivates me toward self-awareness and methodological rigor. I have realized over the years that understanding my shortcomings only makes me more like those I admire and feel so far behind. However, while being aware of your weaknesses is useful for suppressing egotism and its degenerative effects on good science, the greater challenge may be trusting your strengths, such that you may shift the weight of your demands to those areas in which you are most able to bear it. I believe that anyone is ultimately capable of learning anything, and that much suffering could be avoided if more people, particularly those committed to studentship, believed it too.

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I dedicate this thesis to my family, for demonstrating the purpose and meaning of life… and for relinquishing painful sums of money during my dependent years. I will do my best to put your investment to good use. My hope is to avoid a career of brickmaking, and that my effort will not contribute to chaos in the brickyard, but rather to the construction of useful edifices (Forscher, 1963).

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Contributions

Sources of data varied by study and have been cited in the methods section of each chapter. The author (Daniel Felsky) performed all experiments as outlined in each chapter, with the following exceptions:

Chapter 3: Magnetic Resonance Imaging (MRI) data was collected by scan technicians at Toronto General Hospital (TGH) as part of a larger study recruiting out of the Centre for Addiction and Mental Health (CAMH). Whole brain tractography and fibre clustering for some subjects was performed by Dr. Aristotle Voineskos.

Chapter 4: MRI data were collected independently at TGH and Zucker Hillside Hospital (ZHH, Glen Oaks, NY, USA) by local scan technicians. Preprocessing of MRI data from ZHH was performed by Dr. Philip Sczezko and Dr. Toshikazu Ikuta. Neuropathological assessments in the Religious Orders Study (ROS) and Memory and Aging Project (MAP) were overseen by Dr. Julie A. Schneider. Preprocessing and initial summary statistical analyses of postmortem neuropathology data were performed by Dr. Lei Yu.

Chapter 5: Alzheimer's Disease Neuroimaging Initiative (ADNI) data were acquired at multiple sites across the United States and Canada, and are stored and maintained in the Laboratory of Neuro Imaging (LONI) Image Data Archive (IDA) at University of Southern California (Los Angeles, CA). Clinical, imaging, and genetic ROS/MAP data are stored and maintained at the Rush Alzheimer's Disease Center (RADC) at the Rush University Medical Center (Chicago, IL, USA). Neuropathological assessments were overseen by Dr. Julie A. Schneider. Microglia counts were obtained by trained research assistants and post-doctoral fellows at Rush University Medical Center. ROS/MAP genomic imputation was overseen by Dr. Philip L. De Jager. ROS/MAP MRI data were gathered as part of an imaging sub-study run by Drs. Zoe Arvanitakis and Konstantinos Arfaniakis. Dr. Debra Fleischman contributed blood-based biomarker data.

Chapter 6: RNA sequencing data were preprocessed and compiled by Jishu Xu. Sources of neuroimaging data for ADNI and ROS/MAP datasets were the same as from Chapter 5.

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List of Abbreviations

A4 Trial	Anti-Amyloid Treatment in Asymptomatic Alzheimer's Disease Trial
AA	African American
Aβ	beta amyloid
ACh	acetylcholine
AChEI	acetylcholinesterase inhibitor
AD	Alzheimer's disease
ADAS	Alzheimer's Disease Assessment Scale
ADC	apparent diffusion coefficient
ADCS	Alzheimer's Disease Cooperative Study
ADGC	Alzheimer's Disease Genetics Consortium
ADNI	Alzheimer's Disease Neuroimaging Initiative
ADRC	Alzheimer's Disease Research Center
ADRDA	Alzheimer's Disease and Related Disorders Association
ADSP	Alzheimer's Disease Sequencing Project
AF	arcuate fasciculus
AIBL	Australian Imaging, Biomarker and Lifestyle Flagship Study of Ageing
a-MCI	amnestic mild cognitive impairment
ANCOVA	analysis of covariance
API	Alzheimer's Prevention Initiative
APOE	apolipoprotein E
APOJ	apolipoprotein J (clusterin)
APP	amyloid precursor protein
ASC	Alzheimer's Society of Canada
AxD	axial diffusivity

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BA	Brodmann area
BACE1	beta-site APP-cleaving enzyme 1
BBB	blood-brain barrier
BDNF	brain-derived neurotrophic factor
BMI	body mass index
CAA	cerebral amyloid angiopathy
CAMH	Centre for Addiction and Mental Health
CAIDE	Cardiovascular Risk Factors, Aging and Dementia
CB	cingulum bundle
CBRS	CERAD Behavioral Rating Scale
CC	corpus callosum
cdk5	cyclin-dependent kinase-5
CDR-SB	Clinical Dementia Rating Sum of Boxes
CERAD	Consortium to Establish a Registry for Alzheimer's Disease
ChAT	choline acetyltransferase
CHS	Cardiovascular Health Study
CI	confidence interval
CIRS-G	cumulative illness rating scale – geriatric
CLU	clusterin
CN	cognitively normal
CNS	central nervous system
CNV	copy number variant
CRP	C-reactive protein
CSF	cerebrospinal fluid
CT	computed tomography
DALY	disability-adjusted life-years

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dbGaP	database of Genotypes and Phenotypes
DIAN	Dominantly Inherited Alzheimer's Network
DLB	dementia with Lewy bodies
DSI	diffusion spectrum imaging
DSM-5	Diagnostic and Statistical Manual, fifth edition
DTI	diffusion tensor imaging
EEG	electroencephalography
eQTL	expression quantitative trait locus
ELISA	enzyme-linked immunosorbent assay
EMA	European Medicines Agency
ENCODE	Encyclopedia of DNA Elements
ENIGMA	Enhancing Neuro Imaging Genetics through Meta-Analysis
EOAD	early-onset Alzheimer's disease
ERAP1	endoplasmic reticulum aminopeptidase 1
FA	fractional anisotropy
FAD	familial Alzheimer's disease
FAST	Functional Assessment Staging
FDA	Food and Drug Administration
FDG	Fludeoxyglucose
FDR	false discovery rate
FINGER	Finnish Geriatric Intervention Study to Prevent Cognitive Impairment and Disability
FLAIR	fluid-attenuated inversion recovery
fMRI	functional magnetic resonance imaging
FMRIB	Oxford Centre for Functional Magnetic Resonance Imaging of the Brain
FPKM	fragments per kilobase of exon per million reads mapped

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FSL	FMRIB software library
FTD	fronto-temporal dementia
FTDP-17	fronto-temporal dementia with parkinsonism-17
FWE	family-wise error
GAP-43	growth-associated protein 43
GCC	genu of corpus callosum
GINA	Genetic Information Nondiscrimination Act
GM-CSF	granulocyte macrophage colony-stimulating factor
GSK-3	glycogen synthase kinase 3
GTEx	Genotype-Tissue Expression
GWAS	genome-wide association study
HAB	high-affinity binder
HARDI	high angular resolution diffusion imaging
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HRT	hormone replacement therapy
HSV-1	herpes simplex virus type 1
IATI	INNOTEST® Amyloid Tau Index
ICV	intra-cranial volume
IFNγ	interferon gamma
IFOF	inferior fronto-occipital fasciculus
IGAP	International Genomics of Alzheimer's Project
IL	interleukin
ILF	inferior longitudinal fasciculus
iPSC	induced pluripotent stem cell
IWG	International Working Group

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kDa	kilodalton
KEEPS	Kronos Early Estrogen Prevention Study
LAB	low-affinity binder
LD	linkage disequilibrium
LPA	logopenic progressive aphasia
MAB	medium-affinity binder
MAF	minor allele frequency
MAO-B	monoamine oxidase B
MAP	Memory and Aging Project
MAPT	Multidomain Alzheimer Prevention Trial
MCI	mild cognitive impairment
MD	mean diffusivity
MHC	major histocompatibility complex
MMSE	Mini Mental Status Exam
MRC-CFAS	Medical Research Council Cognitive Function and Ageing Study
MRI	magnetic resonance imaging
na-MCI	non-amnestic mild cognitive impairment
NFT	neurofibrillary tangle
NIA	National Institute on Aging
NIH-AA	National Institutes of Health – Alzheimer's Association
NINCDS	National Institute of Neurological and Communicative Disorders and Stroke
NINDS	National Institute of Neurological Disorders and Stroke
NMDAR	N-methyl D-aspartate receptor
NODDI	neurite orientation dispersion and density imaging
NSAID	non-steroidal anti-inflammatory drug

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ODI	orientation dispersion index
OLS	ordinary least squares
OR	odds ratio
PACt-MD	Preventing Alzheimer's dementia with Cognitive remediation plus tDCS in MCI and Depression
PBR	peripheral benzodiazepine receptor
PCR	polymerase chain reaction
PET	positron emission tomography
PHFtau	paired helical filament tau
PIB	Pittsburgh compound B
PMI	postmortem interval
PreDIVA	Prevention of Dementia by Intensive Vascular Care
PSEN1	presenillin-1
PSEN2	presenilin-2
PUFA	polyunsaturated fatty acid
QBI	Q-ball imaging
QTL	quantitative trait locus
RBANS	repeatable battery for the assessment of neuropsychological status
RD	radial diffusivity
RFLP	restriction fragment length polymorphism
RIN	RNA integrity number
ROI	region of interest
ROS	Religious Orders Study
SCC	splenium of corpus callosum
SES	socioeconomic status
SLF	superior longitudinal fasciculus

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SMC	significant memory concern
SNP	single nucleotide polymorphism
SORCS3	sortilin-related VPS10 domain containing receptor 3
SORL1	sortilin-related receptor, L(DLR class), A repeats containing
SORT1	sortilin 1
SPECT	single-photon emission computed tomography
SS	sagittal stratum
SVD	small vessel disease
TARCC	Texas Alzheimer's Research and Care Consortium
TOMM40	translocase of outer mitochondrial membrane 40
TBI	traumatic brain injury
TBSS	tract-based spatial statistics
TBV	total brain volume
TDCS	transcranial direct current stimulation
TDP-43	TAR DNA-binding protein 43
TE	echo time
TFCE	threshold-free cluster enhancement
TGF-β	transforming growth factor beta
TMS	transcranial magnetic stimulation
TNFα	tumor necrosis factor alpha
TR	repeat time
TREM2	triggering receptor expressed on myeloid cells 2
TSPO	translocator protein
UCSC	University of California, Santa Cruz
UF	uncinate fasciculus
VaD	vascular dementia

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VEGF	vascular endothelial growth factor
VLDL	very low density lipoprotein
VLP-1	visinin-like protein 1
VPS10d	vacuolar protein sorting 10 domain
WAIS	Weschler Adult Intelligence Scale
WHI-MS	Women's Health Inititative Memory Study
WHO	World Health Organization
WMH	white matter hyperintensity
YLD	years lost to disability
YLL	years of life lost
ZHH	Zucker Hillside Hospital

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Chapter 1

1 Literature Review

1.1 Alzheimer's Disease

1.1.1 Discovery and Early History

The neuropsychiatric term "Alzheimer's disease" (AD) has only existed since the early 20th century, however, the medicophilisophical concept of dementia – essentially a general loss of capacity to act or reason responsibly – has existed since ancient times. Perhaps the first description of dementia came from Pharaonic Egypt, around 900 BC, where the Maxims of Ptah Hoty described senility as a second childhood (Roman, 2002):

> *My sovereign master, old age is here. Senility has descended on me… my spirit is forgetful and I can no longer remember yesterday*

Up until the late 19th century, dementia was accepted as merely an exaggeration of the aging process (hence "senile dementia"), with known clinical presentation of progressive memory loss, changes in mood, and impairments in speech and executive function. This understanding was mostly fueled by macroscopic investigations of the postmortem brain, showing diffuse signs of deterioration in texture and size similarly in dementia and old age.

In 1893, a Czech neurologist and psychiatrist named Arnold Pick (known for identifying Pick's disease, now known as frontotemporal dementia (FTD) (Kertesz & Munoz, 1998; Pick, 1906)) recognized that focal atrophic brain changes may lead to specific cognitive disturbances observed in senile dementia. At the same time, Paul Blocq and Georges Marinesco described 'amas ronds' (round heaps) in the cortex of an elderly epileptic patient that stained more strongly than the surrounding neutropil (Blocq & Marinesco, 1892). Only a few years later, Emil Redlich first observed sclerotic plaques in a case of senile dementia (Redlich, 1898), assuming them to be proliferated glial cells. In 1910, Oskar Fisher identified the plaques as deposits of indeterminate nature. As such, before the publication of now-famous research by its namesake, neither the clinical presentation nor plaque neuropathology of what we now call AD were unknown.

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What is regarded as the unearthing of the neuropathological characteristics of AD as we know it today began as the description of "a peculiar severe disease process of the cerebral cortex" by the German psychiatrist and neuroanatomist Alois Alzheimer (Hippius & Neundörfer, 2003). Alzheimer, a colleague of Emil Kraepelin (who coined the term "dementia praecox" to describe what is now known as schizophrenia) had witnessed the symptomatological progression of patient Auguste Deter after she was admitted to hospital for untreatable paranoia. Her condition quickly worsened to include sleep disturbances, memory loss, progressive confusion, and ultimately her death five years later in April, 1906. Alzheimer's examination of her brain at autopsy found the senile plaques ("miliary bodies") and neurofibrillary tangles (NFT) ("dense bundles of fibrils") that today constitute the neuropathological signature of AD (Alzheimer, Stelzmann, Schnitzlein, & Murtagh, 1995; Schachter & Davis, 2000). While the plaques had been observed years earlier (Redlich, 1898), Alzheimer was the first to identify neurofibrillary tangles using silver staining. It was Emil Kraepelin who named the illness "Alzheimer's disease" when he included it in the 8th edition of his textbook *Psychiatrie* in 1910 (Hippius & Neundörfer, 2003); it has been suggested that Kraepelin coined the term mainly to attract attention and prestige to his Munich laboratory (in competition with Arnold Pick and Oskar Fisher in Prague) (Beach, 1987) and that Alzheimer never intended to identify a homogeneous disease process but rather to contribute to the basic understanding of neurobiology (Berrios, 1990; Maurer, Volk, & Gerbaldo, 1997).

It wasn't until the late 1960s that clinical neuropathologists showed the plaque and tangle pathology characteristic of AD was found often in old age (in subjects both with (Tomlinson, Blessed, & Roth, 1970) and without (Tomlinson, Blessed, & Roth, 1968) dementia) and likely the most common cause of dementia in elderly (Roth, Tomlinson, & Blessed, 1966). In 1976, Robert Katzman published a noteworthy editorial highlighting the insidious nature of AD pathology and the fact that despite its lack of acknowledgement as a cause of death in society, it likely ranked among the most prevalent (Katzman, 1976). He accurately predicted that AD would present a major socioeconomic burden as the population ages. Public recognition of the threat of AD progressed over the next several decades, which saw the establishment of the National Institutes on Aging (NIA, Oct 7, 1974), the emergence of international criteria and standardized pathologic procedures for AD diagnosis (Mirra et al., 1991; Moms et al., 1989), and ultimately the solidification of the neuropathological identity of AD.

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While today AD is an accepted diagnostic term, the development of its nomenclature has partly evolved from a convolution of nosological and pathophysiological concepts, as well as unique political circumstances surrounding its discovery at the turn of the 20th century. As a result of technological advances in the modern scientific era, much work has been done to disentangle and properly define the characteristic pathologies and symptoms that are encompassed under the term AD. As such, a sort of renaissance is under way, whereby AD is understood to be a spectrum of disease rather than a single process (McKhann et al., 2011). A perspective rooted in the history of the disease may help shed some light on the immense heterogeneity of the modern AD diagnosis and provides century-old impetus for much of the work presented in this thesis.

1.1.2 Diagnosis

In 2010 and 2011, the International Working Group (IWG) (Dubois et al., 2010) and National Institutes of Health-Alzheimer's Association (NIH-AA) working group (McKhann et al., 2011) met to re-evaluate and update earlier AD diagnostic criteria (McKhann et al., 1984) based on the rapidly-evolving research landscape. Both groups recognized the value of objective biomarkers (fluid and imaging) in reaching diagnosis (in fact, the IWG guidelines require biomarker abnormalities for diagnosis); interestingly, the NIH-AA guidelines permit a diagnosis of possible AD in the absence of core AD cognitive symptoms ("possible AD with evidence of the AD pathophysiological process"). Most recently, in 2013, the Diagnostic and Statistical Manual, Fifth Edition (DSM-5) (American Psychiatric Association, 2013), the authoritative psychiatric classification and diagnostic tool, was published. In the DSM-5, dementia was reclassified to major and minor neurocognitive impairment; to meet criteria for major cognitive impairment the individual must show core cognitive deficits that significantly impair functional independence in everyday activities, whereas the minor type refers to situations where changes in cognition have not yet impacted everyday function. Despite this new proposed nomenclature, the term "dementia" remains prevalent in the literature and will be used throughout this thesis.

Currently, the diagnostic process for AD in living humans is somewhat complex and involves both objective and subjective assessments of an individual's daily functioning and cognitive performance over time. From McKhann et al., 2011, a clinical diagnoses of AD must be made on a background diagnosis of dementia, which requires that an individual 1) be experiencing

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cognitive or behavioural symptoms that interfere with daily functioning, 2) have demonstrated a decline in performance or functioning from a previous state, and 3) show symptoms that are not explained by delirium or a major psychiatric disorder. Further, there are specific requirements for which cognitive domains must be impaired to warrant a diagnosis of dementia; learning, reasoning, visuospatial abilities, language, and personality, behavior or comportment. In cases where the individual's ability to function is not significantly impaired (by the judgement of an experienced clinician, the patient, and a knowledgeable informant), a diagnosis of mild cognitive impairment (MCI) may be more appropriate. Following the diagnosis of dementia comes the ascertainment of its likely source; the DSM-5 lists 13 potential underlying causes for major neurocognitive disorder including AD, Lewy body disease, HIV infection, traumatic brain injury, substance use, Huntington's disease, and "unspecified" (American Psychiatric Association, 2013).

Clinical AD may be diagnosed as A) probable, B) possible, or C) probable or possible AD with evidence of the AD pathophysiological process (McKhann et al., 2011). Only A and B are intended for use in clinical settings, whereas C is intended for research purposes. In the case of A) probable AD, the subject meets criteria for dementia as well as 1) shows insidious onset (months to years, rather than hours to days), 2) has a clear history of worsening cognition, and 3) has a prominent amnestic presentation or most prominent cognitive deficits in language, visuospatial presentation, or executive dysfunction. AD diagnosis is excluded if there is evidence of cerebrovascular disease or other dementias/aphasias. If the subject possesses genetic mutations known to cause early-onset Alzheimer's disease (*APP*, *PSEN1*, or *PSEN2*), or a well-documented history of cognitive decline, then the diagnosis can be upgraded to probable AD with "increased level of certainty". A diagnosis of possible AD can be made in the event that a subject displays the core cognitive deficits found in AD, but has an atypical course (e.g. sudden onset) or etiologically mixed presentation (e.g. concomitant cerebrovascular disease or features of other dementias). Finally, A definite diagnosis of AD can only be made upon postmortem evaluation of brain tissue; biopsy or autopsy must yield histopathologic evidence of amyloid plaque and neurofibrillary tangle pathology (McKhann et al., 1984). Currently, the definitive criteria for postmortem neuropathological assessment of AD are those according to the NIA-AA, published in 2012 (Montine et al., 2012)

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Given the insidious and gradual onset of AD symptoms, the recognition and definition of the intermediate stage between healthy aging and dementia has been of great interest. The term "mild cognitive impairment" (MCI) was first introduced in 1988 by Reisberg et al. (Reisberg et al., 1988) to refer to this stage, and efforts headed by Ronald Petersen at the Mayo Clinic have subsequently sought to clarify its neuropathological and clinical characteristics (Petersen, 2004; Petersen et al., 1999, 2001, 2009, 2014), leading to the publication of international MCI criteria by the First Key Symposium held in Stockholm, Sweden in 2003 (Winblad et al., 2004). More recently, in two sister publications to the 2011 NIH-AA Work Group paper (McKhann et al., 2011), Albert et al. (Albert et al., 2011) and Sperling et al. (Sperling et al., 2011) aimed to outline the pre-clinical phases of AD by establishing guidelines for the diagnosis of "MCI due to AD" (with varying levels of certainty) and describing the stages of progression in elderly from asymptomatic (and without evidence of the AD pathological process) to MCI. A diagnosis of MCI is made for individuals who demonstrate a degree of cognitive decline in core AD-related domains that is not normal for age, yet who do not fulfil clinical criteria for dementia. As with a diagnosis of AD, it is necessary to rule out other systemic or brain conditions that may account for the clinical and cognitive decline observed in MCI (e.g. vascular, traumatic, or medical). MCI may be either amnestic (a-MCI; showing significant memory impairment) or non-amnestic (na-MCI; no significant memory concerns), and cognitive impairment for individuals in either group may be further classified as single- or multi-domain in nature (Winblad et al., 2004). Importantly, the diagnosis of MCI is not stable; in a multiethnic study of 2 364 individuals aged 65 and over, only 23% of those with MCI progressed to AD and 31% reverted to normal levels of cognition, with a-MCI subjects having greatest risk of progression and lowest for reversion (Manly et al., 2008).

1.1.3 Heterogenety and Subtypes

Before discussing AD further as a single entity, for the purposes of this thesis it is necessary to define it within the context of its various forms. For the remainder of this thesis, the acronym "AD" will refer specifically to the late-onset (sporadic) form of disease, with age-at-onset after 65 years of age. There also exist early-onset manifestations of AD (herein EOAD), which includes the rare autosomal dominant familial AD (FAD, which accounts for less than 1% of all

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AD cases and is driven by several heritable and highly penetrant mutations in key amyloid-related genes (St. George-Hyslop et al., 1987)), which is defined only by an earlier age-at-onset of before 65 (Wu et al., 2012). Though the neuropathological features of AD and EOAD are thought to be shared, active research is pursuing the relationship between age-at-onset of AD and disease etiology, progression, and mortality.

Based on non-specificity of diagnostic criteria alone, the heterogeneity in clinical presentation of AD is understandably great. However, when one considers the range of etiopathologies and individual progression profiles of those diagnosed with AD, the story becomes considerably more complex. The phenotypic heterogeneity of AD has been recognized for over half a century; in 1969, Carrick McDonald differentiated a group of 57 female AD patients into a "parietal group", featuring primarily praxis, visual construction, and cortical sensation deficits, and a "benign memory dysfunction of aging" group, who had predominant memory dysfunction, later age-at-onset, and slower disease progression (C. McDonald, 1969). In the 1980s, Martin et al. suggested that AD pathology may lead to multiple distinct neuropsychological syndromes (Martin et al., 1986), eroding the then widely-held belief that subtypes of AD were just mislabeled cross-sectional observations of a singular AD process at different stages (Ritchie & Touchon, 1992). Subsequently, Fisher et al. demonstrated that three distinct neuropsychologically-defined subtypes of AD (termed global, right, and left) had the same age-at-onset (N. J. Fisher et al., 1996) and were longitudinally stable (N. J. Fisher et al., 1997), which helped solidify the concept of truly distinct AD subtypes. More recently, it has been recognized that AD may present with more focal symptoms resulting from regionally-specific pathologies (Kramer & Miller, 2000), and that neuropathologically-defined subtypes of AD can be consistently identified based on the relative locations and extents of neuropathological lesions (Murray et al., 2011).

Subtyping efforts have mostly focused on either symptoms (more subjective) or neuropathology (objective). Neuropsychiatric symptoms, which are experienced by 80-90% of AD subjects (with estimates as high as 97%) (Steinberg et al., 2004, 2008) and include behavioural, psychotic, and mood disturbances (Lyketsos et al., 2011), have also commonly been used to identify AD subtypes. Recently, eight separate cognitive subtypes of AD were identified using latent class analysis; in 938 probable AD patients, these cognitive subtype clusters were correlated with neurobiological and demographic markers (N. M. E. Scheltens et al., 2015). As recently

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reviewed by Nowrangi et al. (Nowrangi, Lyketsos, & Rosenberg, 2015), the literature on groupings according to neuropsychiatric symptoms most often identifies three clusters of subjects; those experiencing 1) behavioural dysfunction, 2) psychosis, and 3) mood disturbance. Identifying the neuronal circuitry underlying these complex symptoms is an active area of investigation. Due to disparity in methodologies used to assess symptoms and pathologies, results of subtyping efforts across studies have largely not unified; however, a few subtypes of AD patients who may represent distinct underlying disease processes have been more reliably identified by multiple lines of evidence, and they are described below.

A set of AD subtypes with convergent lines of evidence (symptoms and neuropathology), recently summarized by Lam et al. (Lam, Masellis, Freedman, Stuss, & Black, 2013), include: typical AD (aka classic onset; predominantly amnestic, associated with hippocampal and temporo-parietal atrophy and/or decreased perfusion/metabolism – 6% of typical AD subjects present with early onset, compared to 32% of non-typical (Koedam et al., 2010)), temporal variant AD (aka focal temporal lobe dysfuntion or pure amnestic AD), left (language) variant AD (similar to logopenic progressive aphasia, which is often associated with AD pathology (Alladi et al., 2007)), right (visuoperceptive) variant AD, and frontal variant AD. Two additional subtypes, hippocampal sparing and limbic predominant AD, have also gained recent traction (Murray et al., 2011; Jennifer L Whitwell et al., 2012), however these are not mutually exclusive of the categories listed above and show overlap with left variant and temporal variant AD, respectively (Lam et al., 2013).

Typical AD is the most common presentation of AD. It is characterized by a late onset (age 65+), steady and uniform decline in cognitive function across domains, and symmetric and relatively non-specific patterns of brain atrophy and hypometabolism/hypoperfusion (though regions most affected are entorhinal, hippocampal and temporo-parietal) (Dubois et al., 2007; McKhann et al., 2011).

Temporal variant AD is defined by a late onset (later than typical AD), isolated impairment in episodic memory performance (with sparing of other cognitive domains), and a slow progression (Butters, Lopez, & Becker, 1996); in fact, subjects presenting with focal temporal lobe dysfunction show little to no longitudinal change in cognition over two years (Marra et al., 2012). Aligning with observations of subgroups of AD patients with plaque and NFT pathology

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restricted to limbic regions (Armstrong, Nochlin, & Bird, 2000), temporal variant AD likely overlaps significantly with neuropathologically-defined **limbic predominant AD** (Murray et al., 2011). Of particular interest for work in this thesis, it is possible that temporal variant AD subjects have greater cognitive reserve than typical and other atypical forms of AD; hence their later age-at-onset, slower rates of decline, and relative sparing of cortical degeneration.

Left variant AD is characterized by the presentation of marked language-specific deficits that are inconsistent with typical AD. As temporal variant is memory-specific and involves the focal deterioration of limbic regions, left variant is specific to worsening non-fluent speech and atrophy of the left perisylvian region with relative sparing of the hippocampus/limbic regions (hence overlap with hippocampal sparing AD) (Galton, Patterson, Xuereb, & Hodges, 2000; Greene, Patterson, Xuereb, & Hodges, 1996; J. Green, Morris, Sandson, McKeel, & Miller, 1990). The non-fluency of speech in left variant AD is a distinguishing feature from both typical AD and the closely related logopenic progressive aphasia (LPA); late-stage typical AD and LPA both show more fluent language syndromes (including slowed rate of speech, semantic paraphasia (erroneous substitution/use/omission of related words), impaired repetition (commonly in LPA), and dyslexia) compared to left variant AD-associated agrammatism and phenomic paraphasia (erroneous substitution/use/omission of similar-sounding words) (Gorno-Tempini et al., 2008; B. H. Price et al., 1993).

Right variant AD is characterized by the presentation of strong visuospatial dysfunction compared to other more typical deficits, and is associated with posterior cortical atrophy (Tang-Wai et al., 2004). The name of right variant AD comes from the longitudinal stability of distinct asymmetries in neuropathology and cortical atrophy that affect the right hemisphere to a greater degree than left (Duara et al., 1986, 1986; N. J. Fisher et al., 1997). This subtype was first suggested by Chase et al. (Chase et al., 1984), who found correlation between Wechsler Adult Intelligence Scale (WAIS) visuospatial subtest scores and right hemisphere metabolism (measured by [18F]Fluorodeoxyglucose (FDG) positron emission tomography (PET) imaging (discussed in Section 1.3.4)) in 17 AD subjects.

Frontal variant AD is a rare type of EOAD (total 5 frontal variant AD/163 total AD cases (3%) across two studies (Alladi et al., 2007; Johnson, Head, Kim, Starr, & Cotman, 1999)) associated with deficits in executive function in the relative absence of typical amnestic symptoms

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(dysexecutive syndrome), perhaps driven by a ten-fold increase in NFTs in frontal regions (Johnson et al., 1999), as well as behavioural symptoms (Alladi et al., 2007). Recently, it was proposed that frontal variant AD be renamed to "the behavioural/dysexecutive variant of AD" based on observations that frontal gray matter is in fact spared in these subjects (Ossenkoppele et al., 2015).

Hippocampal sparing AD was first observed in 1994 (Giannakopoulos, Hof, & Bouras, 1994) and involves the disproportionate atrophy of cerebral cortex compared to the hippocampus, which is affected to a greater degree in typical AD. Based on their neuropathological definitions, it is possible that the hippocampal sparing AD and limbic predominant AD subtypes represent distinct etiological processes rather than just neuroanatomical variations on the same process; recently, Josephs et al. (Josephs et al., 2015) found that TAR DNA-binding protein 43 (TDP-43, which is found in 19-57% of AD cases, contributes to AD cognitive deficits (Josephs, Whitwell, et al., 2014) and progresses topologically in stages (Josephs, Murray, et al., 2014), much like NFTs (H. Braak & Braak, 1997)) deposition was common in typical (59%) and limbic (67%), but not hippocampal sparing (21%) AD pathological subtypes; though clinical presentation was only affected by pathological subtype, not TDP-43.

Efforts to subtype AD based on genetic information have largely consisted of understanding the differential contributions of the ε4 allele of the Apolipoprotein E gene (*APOE*) (Section 1.4.4). Because this gene variant has been found to strongly influence risk for AD (Corder et al., 1993), it has been of interest to examine ε4 carrier status in individuals who develop AD and look for differences in presentation and progression between groups. Generally, *APOE* ε4 is associated with amnestic presentation (as opposed to more frontal-based, aphasic, early-onset form) (Snowden et al., 2007). However, some evidence suggests that *APOE* ε4 is underrepresented in individuals with temporal variant AD (Butters et al., 1996), despite its association with hippocampal atrophy (Pievani et al., 2011). The effects of genetic variation on AD presentation and clinical/biological phenotypes will be discussed in-depth in Section 1.4. The contribution of genetics to AD heterogeneity is not well understood.

In summary, it should be apparent that the aforementioned AD subtypes, while potentially representing distinct underlying disease processes, lie on a continuous spectrum that relies on

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age-at-onset, co-morbid pathologies, neuropathological topology, genetic risk factors, and clinical presentation to determine and define their identities.

1.1.4 Prevalence and Economic Impact

AD is the most common form of dementia, accounting for 60-80% of all cases (Alzheimer's Association, 2015; Barker et al., 2002), and it is estimated that currently there are 47.5 million people living with dementia worldwide (World Health Organization, 2015). Ferri et al. (Ferri et al., 2005) estimated that in 2005, 24 million individuals worldwide had dementia and that by 2020 and 2040 this number would increase to 42 and 81 million, respectively. They also made observations that the prevalence and incidence rates in different areas of the world were not consistent; developed countries showed the highest prevalence but a slower predicted increase (100% between 2001-2040) compared to developing nations that had lower prevalence but greater predicted increase (up to 336% by 2040).

To allow for forecasting, Brookmeyer et al. (Brookmeyer, Johnson, Ziegler-Graham, & Arrighi, 2007) developed a multistate model of AD (including AD-specific disease progression and incidence rates, as well as transition probabilities between healthy and early vs. late stage AD) using population projections from the United Nations. They estimated that between 2006 and 2050 the worldwide prevalence of AD would grow fourfold (from 26.6 million to 106.8 million). Similar to the results of Ferri et al., they found that the prevalence of AD increases exponentially with age, however, importantly, the estimates of Brookmeyer et al. were specific to AD rather than all dementia. Furthermore, using their multistate model, they were able to show that if the age-at-onset of AD were to be delayed by only two years, there would be approximately 22.8 million fewer cases of disease worldwide by 2050.

Most estimates of AD prevalence are based on static parameters of its incidence over time. The literature on rates of AD and dementia incidence, however, is heterogeneous, with studies describing increases (Ukraintseva, Sloan, Arbeev, & Yashin, 2006), no significant changes (Beard, Kokmen, Offord, & Kurland, 1991; Hebert et al., 2010; Rocca, Cha, Waring, & Kokmen, 1998; Rorsman, Hagnell, & Lanke, 1986), and decreases (Hagnell, Lanke, Rorsman, Ohman, & Ojesjö, 1983; Kokmen, Beard, O'Brien, Offord, & Kurland, 1993; Rorsman et al., 1986) in the

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rate of AD diagnosis over time. Some have asserted that prevalence estimates based on static risk remain valid, given methodological discrepancies that may explain some of this heterogeneity (Hebert et al., 2010). However, following these assertions, three recent reports suggested that the incidence of dementia is actually decreasing, likely due to modern advances in treating vascular disease and increasing levels of education (Matthews et al., 2013; Qiu, von Strauss, Bäckman, Winblad, & Fratiglioni, 2013; Schrijvers et al., 2012).

In recent reports of AD prevalence worldwide and in the United States and Canada (Alzheimer's Association, 2015; The Alzheimer Society of Canada, 2010; World Health Organization and Alzheimer's Disease International, 2012), the message is the same: dementia and AD (either specifically or indirectly as the number one cause of dementia) are imminent global healthcare burdens, largely due to an aging population, with steadily increasing prevalence and dramatic costs that outweigh those forecasted for other major causes of disability and death. A recent systematic review and meta-analysis conducted by Prince et al. (Prince et al., 2013) found that the number of individuals worldwide living with dementia is expected to nearly double every 20 years from 35.6 million in 2010 to 115.4 million in 2050, with the proportion of cases in low-middle income countries growing from 58% to 71% over the same interval.

As a result of increasing AD prevalence, rates of AD-related mortality and disability are expected to rise. Indeed this trend has already begun, and due to a lack of effective treatments for AD, the rise in mortality and loss of quality/duration of life is disproportionately large compared to other major diseases. In the United States, between 2000 and 2013, the percent changes in causes of death (at all ages) for breast and prostate cancer, heart disease, stroke, and HIV were negative (from between -2% to -52%), whereas that for AD was +71% (National Center for Health Statistics, 2015). A statistic used by the World Health Organization (WHO) to assess the loss of quality and/or length of life due to AD is disability-adjusted life-years (DALY), which is equal to years of life lost (YLL = number of deaths x standard life expectancy at age of death) + years lost to disability (YLD = number of cases x disability weight x average duration of illness). Using this composite metric, it was found that AD rose from the 25th most burdensome disease in the United States in 1990 to 12th in 2010, a greater rise than any other disease (Alzheimer's Association, 2015). During the same period, and considering YLL alone, AD rose from 32nd to 9th; again, the largest rank increase.

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Both direct and indirect costs of dementia and AD are alarmingly high. Direct costs represent resources used in the care of patients with dementia (increasing with loss of autonomy and need for hospitalization and informal care), whereas indirect costs encompass those resources lost as a result of reduced productivity or work absenteeism on the part of either the patient or their family/caregivers (Davidson & Schnaider Beeri, 2000). In total, the worldwide cost of dementia was estimated to be US$315.4 billion (based on an estimate of 29.3 million affected) in 2005 (Wimo, Winblad, & Jönsson, 2007) and US$422 billion (based on 34.4 million affected) in 2009 (Wimo, Winblad, & Jönsson, 2010). This number rose to US$604 billion in 2010 (World Health Organization and Alzheimer's Disease International, 2012). In Canada alone, total costs of dementia are estimated to rise from CA$14.9 billion in 2008 to CA$152.6 billion by 2038, within one generation (The Alzheimer Society of Canada, 2010). Clearly, based on dramatically increasing prevalence and costs of AD worldwide there is a need for understanding its modifiable risk factors and developing effective interventions for those we cannot control: delaying the onset of AD by only two years would result in a savings of CA$15 billion in health care to Canadians over a period of one decade (The Alzheimer Society of Canada, 2010).

1.1.5 Progression, Risk, and Intervention

Since there is currently no cure for AD and the process is progressive and degenerative, the prognosis for an individual diagnosed with AD is grim. The estimated median survival time in individuals who develop AD is 7.1 years (95% confidence interval (CI) = 6.7-7.5 years) (Fitzpatrick, Kuller, Lopez, Kawas, & Jagust, 2005), though individual survival estimates are quite variable and usually between 4-8 years after diagnosis (World Health Organization and Alzheimer's Disease International, 2012; J. Xie, Brayne, & Matthews, 2008); cause of death is not easily attributable to AD, as many patients die of co-morbid conditions or accidents and dementia is under-reported (Ganguli & Rodriguez, 1999; Romero, Benito-León, Mitchell, Trincado, & Bermejo-Pareja, 2014).

In a study using functional assessment staging (FAST) to measure rates of decline in 648 AD patients (Thalhauser & Komarova, 2012), it was found that rates of decline generally followed two distinct distributions, fast and slow, which were stable over time (i.e. a patient progressing slowly initially would continue to progress slowly). Factors contributing to rates of disease

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progression as measured by cognitive decline include psychotic symptoms, aggressive behaviour, sleep disturbance, and depressive symptoms (Komarova & Thalhauser, 2011; Mortimer, Ebbitt, Jun, & Finch, 1992; Schmeidler, Mohs, & Aryan, 1998; Storandt, Grant, Miller, & Morris, 2002; Zahodne, Ornstein, Cosentino, Devanand, & Stern, 2015). Over the 11-year course of the Cache County Dementia Progression Study (DPS) (Tschanz et al., 2011; Tschanz, Norton, Zandi, & Lyketsos, 2013), AD subjects with a history of atrial fibrillation, systolic hypertension, and angina showed more rapid rates of cognitive (Mini Mental Status Exam (MMSE) scores) and functional decline (Clinical Dementia Rating Sum of Boxes (CDR-SB)). Generally, poor overall medical health at baseline has also been associated with more rapidly-progressing symptoms (Leoutsakos et al., 2012). It was also found that myocardial infarction predicted faster rates of cognitive decline in individuals possessing the *APOE* ε4 genetic risk factor (M. M. Mielke et al., 2011). The literature on progression of cognitive symptoms in AD as they relates to *APOE* genotype independently, however, is not consistent; studies have suggested both a slower decline (Frisoni et al., 1995) and much faster (non-linear) decline (as well as earlier age-at-onset) (Martins, Oulhaj, de Jager, & Williams, 2005) are associated with *APOE* ε4 status, and that earlier onset patients progress more rapidly without *APOE* ε4 (van der Vlies, 2009). This discrepancy relates to a larger debate over the relationship of neuropathological vs. cognitive progression in AD, which will be discussed more in the context of AD biomarkers (Section 1.3) and genetic risk factors (Section 1.4).

In contrast with those factors that are associated with varying rates of cognitive decline/disease progression, there are several well-known factors that can impact an individual's risk for developing AD. Solomon et al. (Solomon et al., 2014) recently summarized the major risk factors for dementia and AD: increasing age, genetics (*APOE* ε4, other proposed risk alleles), vascular and metabolic conditions (including diabetes, high body mass index (BMI), and high midlife serum cholesterol), lifestyle (smoking and high alcohol intake), diet (saturated fats, homocysteine), depression, traumatic brain injury (TBI), occupational exposure (e.g. heavy metals), and infectious agents (e.g. HIV, Chlamydophila pneumonia). There are also a number of protective factors that are known to reduce an individual's risk for AD including: younger age, genetics (*APOE* ε2, other proposed protective alleles), psychosocial factors (e.g. high education, socioeconomic status (SES), social engagement), lifestyle (physical activity, moderate alcohol intake), diet (e.g. Mediterranean diet, polyunsaturated fatty acids (PUFAs), vitamins B_6, B_{12} and

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D, antioxidants), and drugs (antihypertensive, statins, hormone replacement therapy (HRT), and non-steroidal anti-inflammatory drugs (NSAIDs)).

Not all of these factors are modifiable, and in relation to relevance for treatment, it is important to identify those factors that are subject to modification and assess the costs and benefits of interventions targeting them in at-risk populations. In a recent comprehensive review of 75 longitudinal studies of dementia risk factors, Di Marco et al. (Di Marco et al., 2014) define the key modifiable factors as 1) dietary habits, 2) leisure activities, 3) beverages consumption (including alcohol, juice, tea, and coffee), 4) smoking habits, and 5) social network (including marital status and living arrangements). Sindi et al. (Sindi, Mangialasche, & Kivipelto, 2015) define only three categories of modifiable factors related to AD risk: 1) vascular risk factors (including hypertension, smoking, and stroke), 2) nutrition, and 3) lifestyle and psychosocial factors. Because AD is a late-life disease, cumulative exposure is an important consideration when using risk factors to make predictions or inform clinical trials. For example, hypertension is a known risk factor for AD, but only when observed during mid-life and when taking into account the differing relationships of systolic and diastolic blood pressure with AD (Qiu et al., 2013); the Cardiovascular Risk Factors, Aging and Dementia (CAIDE) Dementia Risk Score was developed to provide 20-year predictions on AD risk for middle-aged individuals based on this (Kivipelto et al., 2006). Another example of the importance of timing came from the Women's Health Initiative Memory Study (WHI-MS) (Shumaker et al., 2003) and the Kronos Early Estrogen Prevention Study (KEEPS) (Tsagkas & Turner, 2012) which administered HRT to women many years after and immediately following menopause onset, respectively. In WHI-MS, the HRT was associated with increased risk for dementia, MCI, and vascular disease, whereas in KEEPS, HRT was protective against vascular disease with no effect on cognition.

Despite many positive studies finding significant effects of modifiable risk factors on AD risk, the literature was heterogeneous, and in 2011, the NIH State-of-the-Science Conference panel evaluated work published on modifiable risk factors and AD prevention between 1984 and 2009, finding that there was insufficient evidence for preventative AD/dementia interventions due to poor quality of available data (Daviglus et al., 2011). Many explanations for the inconsistency in studies looking at potential AD interventions up to 2011 have been proposed including: short intervention durations, late intervention timing, small sample sizes, dosage differences (in cases where medication or nutritional supplements were evaluated), and varying definitions of

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outcome (e.g. dementia, AD, cognition, etc.) (Coley et al., 2008; Mangialasche, Xu, & Kivipelto, 2013). In 2011 and 2013, largely spurred by failures of 12- to 18-month drug trials for AD, and to address the issue of reliable outcome measures in clinical trials as a whole, the European Medicines Agency (EMA) and the Food and Drug Administration (FDA) approved the use of cerebrospinal fluid pathology (Aβ42 and tau protein) and PET (amyloid) as outcome measures to assess efficacy in new trials (Committee for Medicinal Products for Human Use, 2012; Kozauer & Katz, 2013).

Conceptually, treatments and interventions for AD fall into three general categories: alleviating symptoms without affecting the underlying disease process, slowing the progression of disease after onset, and preventing or delaying the onset of disease. For treatment of AD symptoms, there are currently only four medications approved by the FDA for use in the United States and Canada (Craig, Hong, & McDonald, 2011; National Institute on Aging, 2015): donepezil, rivastigmine, galantamine, and memantine. Three of these drugs, donepezil, rivastigmine and galantamine, are known as acetylcholinesterase inhibitors (AChEIs), which act specifically to influence levels of the neurotransmitter acetylcholine, which is depleted in AD (discussed in Section 1.2.2). While these drugs have shown some efficacy in modestly ameliorating symptoms in mild to moderate AD (Lanctôt et al., 2003; Tariot et al., 2000), not all subjects respond and the factors determining differential response are poorly understood (Lazzaro et al., 2005; Van Der Putt, Dineen, Janes, Series, & McShane, 2006). In a meta-analysis of 16 clinical trials, Lanctôt et al. (Lanctôt et al., 2003) found that there was a statistically significant 9% increase in proportion of global responders to treatment using AChEIs vs. placebo, with benefits differing between drugs (donepezil = 3%, galantamine = 14%, and rivastigmine = 8%; though numbers of studies using each drug differed substantially and likely contributed to this difference). The fourth drug, memantine, acts on the glutamate neurotransmitter system, and either alone or in combination with AChEI therapy can be effective in slightly alleviating cognitive and functional impairments in moderate-severe AD (Reisberg et al., 2003), though not in mild AD/MCI (L. S. Schneider, Dagerman, Higgins, & McShane, 2011). Importantly, none of these drugs have been shown to effectively alter the course of disease or delay mortality and some controversy exists over their true clinical value and cost effectiveness (Loveman et al., 2006).

For prevention and delay of onset, epidemiological evidence linking modifiable factors to AD incidence has provided the foundation for a number of clinical trials based on both

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pharmacological and non-pharmacological interventions (Lindsay et al., 2002; Ritchie et al., 2010). Given the aforementioned lack of firm evidence supporting changes to healthcare policy or medical practice (Daviglus et al., 2011), several large intervention trials combining adjustment of multiple modifiable risk factors were initiated, all in non-demented elderly: the Finnish Geriatric Intervention Study to Prevent Cognitive Impairment and Disability (FINGER; n=1 260 subjects, 2 year intervention + 5 year follow-up, completed in 2014) (Kivipelto et al., 2013), the Multidomain Alzheimer Prevention Trial (MAPT; n=1 680 subjects, 3 year intervention + 2 year follow-up, completed in 2014) (Carrié et al., 2012), and the Prevention of Dementia by Intensive Vascular Care (PreDIVA; n=3 533 subjects, 6 year intervention, to be completed in 2015) study (Richard et al., 2009).

Failure has plagued the development of useful preventative AD drugs over the last several decades, with well over 100 failures up to 2008 (Becker, Greig, & Giacobini, 2008). As reviewed in detail by Schneider et al. (L. S. Schneider et al., 2014), the amyloid cascade hypothesis (outlined in Section 1.2.3) has been the primary informant of drug development in AD for the last 20 years. However, recent failures of phase 3 trials of treatments based on the clearance of misfolded amyloid protein (Eli Lily drug solanezumab; Pfizer and Elan drug bapineuzumab) for patients with mild to moderate AD (Fox, 2012; Salloway et al., 2014) have suggested that treatment at this stage (i.e. even at mild AD) is already too late to modify a pathological process that has been gathering momentum over many years. It is now well-accepted that in order to maximize clinical impact, intervention studies should be planned in the presymptomatic stages of AD, using objective molecular biomarkers (Reiman, Langbaum, & Tariot, 2010) and cognitive performance as primary outcome measures. However, to maximize the impact of such trials, specific groups of individuals at high-risk for AD must first be studied, rather than all-comers.

One example of such a group is the large Columbian pedigree (Pastor et al., 2003), with a known mutation in the presenilin-1 (*PSEN1*) gene, who are at high risk for EOAD. Studies suggest that genetic risk carriers vs. non-carriers from this pedigree, ranging from 18-26 years of age (i.e. at least 20 years prior to illness onset), already show differences in magnetic resonsnace imaging (MRI)-based measures of brain structure (Reiman et al., 2012; Sepulveda-Falla, Glatzel, & Lopera, 2012). In that population, it is evident that clinical intervention is required from late adolescence onward to halt or delay neurodegeneration, and a trial sponsored by Genetech, Inc. (Roche; S. San Fanciisco, CA, USA) and the Banner Alzheimer's Institute (Phoenix, AZ, USA)

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called the Alzheimer's Prevention Initiative (API) is currently underway to examine the effects of an anti-amyloid drug (crenezumab) vs. placebo in 300 members of this population over the course of 260 weeks (www.clinicaltrials.gov; NCT019988410). Another major trial with study sites in the United States and Canada, sponsored by the Dominantly Inherited Alzheimer's Network (DIAN) and NIH, is currently recruiting individuals with known AD-causing autosomal-dominant mutations (or a first-degree relative with FAD) to test the efficacy of anti-amyloid therapy (gantenerumab and solanezumab) in preventing AD (NCT01760005; scheduled to end in March 2017). Finally, the Anti-Amyloid Treatment in Asymptomatic Alzheimer's Disease Trial (A4 Trial), headed by Reisa Sperling (Harvard Medical School, Boston, MA, USA), is an international, multi-center trial currently being conducted by the Alzheimer's Disease Cooperative Study (ADCS), with sites in the United States, Canada, and Australia. The goal of the A4 trials is to test the efficacy of solanezumab vs. placebo at preventing AD in 1000 adults between 65 and 85 years of age who have normal cognition yet show brain amyloid accumulation as measured by [18F]Florbetapir PET imaging (Sperling, Rentz, et al., 2014).

The shift in focus from disease-modifying therapies to disease-preventing or -delaying interventions has come largely from our understanding of the process underlying AD as one that is active throughout the lifespan and likely unstoppable once symptoms have emerged. However, the central importance of the misfolded amyloid that is the target of recent trials is not unequivocally supported; substantial evidence implicates other molecules, mechanisms and potentially modifiable pathways in the etiopathogenesis of AD (Section 1.2). In an expansive review, Mangialasche et al. (Mangialasche, Solomon, Winblad, Mecocci, & Kivipelto, 2010) summarize the landscape of AD drug development as of 2010, demonstrating that efforts are underway to test many facets of the AD pathological process including both cholinergic (Section 1.2.2) and non-cholinergic neurotransmission (e.g. dopamine, serotonin, norepinephrine), amyloid production, aggregation, and clearance (Section 1.2.3), tau protein phosphorylation and aggregation (Section 1.2.4), and neuroplasticity (i.e. release of brain-derived-neurotrophic factor (BDNF)). In addition, drug interventions targeting inflammatory processes in AD (Section 1.2.6) have been of great interest due to the availability of approved anti-inflammatory medications, though evidence for their efficacy and safety is not consistent (Jaturapatporn, Isaac, McCleery, & Tabet, 2012; McGeer, Schulzer, & McGeer, 1996).

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1.2 Prominent Hypotheses/Mechanisms of Alzheimer's Disease Etiology and Progression

1.2.1 Background

In order to understand the mechanisms behind known AD risk factors it is essential to first have an understanding of the neuropathological mechanisms at work in AD. Fundamentally, AD is a disorder of synapse loss resulting in brain dysfunction. The number of synapses relative to neurons decreases as the disease progresses and this is the principal biological correlate of disease severity (Selkoe, 2002). There are currently several biochemical pathways and neuropathological mechanisms that are thought to relate to AD risk and/or progression including: loss of acetylcholine-specific neurotransmission (**cholinergic**), beta-amyloid (Aβ) protein accumulation and aggregation (**amyloidogenic**), tau protein hyperphosphorylation and microtubule degeneration (**tauopathic**), cerebrovascular changes (**vascular**), and immune response irregularities/chronic neuroinflammation (**inflammatory**). This section (1.2) aims to describe the events leading to the construction of each hypothesis and summarize our current state of knowledge regarding its involvement in AD.

It should be acknowledged that the list above is not exhaustive; there are many other hypotheses of AD etiology and pathogenesis that have garnered varying degrees of attention over the last several decades. Notably, these include the **glutamatergic** hypothesis (Maragos, Greenamyre, Penney, & Young, 1987), the **oxidative stress** hypothesis (Markesbery, 1997), and the **mitochondrial** cascade hypothesis (Swerdlow & Khan, 2004). While these will not be discussed at length, it is worth mention that the only non-cholinergic pharmacological intervention approved by the FDA for the treatment of moderate-severe AD is memantine, an N-methyl-D-aspartate glutamate receptor (NMDAR) antagonist discovered in 1968 that works by regulating the activity of glutamate. According to the gluatamatergic hypothesis of AD, excitotoxicity of excess glutatmate released from damaged neurons causes the degeneration of pyramidal neurons in the cortex and hippocampus that are important for cognitive function (Butterfield & Pocernich, 2003). Mementine blocks NMDAR binding sites quickly and in a dose-dependent manner, allowing for minimal effects on physiological glutamate signaling which was the cause of intolerable side effects in other NMDAR-blocking drug trials (Lipton, 2006; Thomas & Grossberg, 2009). Mitochondrial dysfunction as it relates to oxidative stress and AD pathology will be discussed within the context of other mechanisms where appropriate.

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An issue with defining distinct models of AD etiopathogenesis is that there exist no real boundaries between these pathways in the brain (e.g. alterations in neurotransmission impact oxidative stress pathways that are intimately tied to vascular changes), and so the definitions of the pathways outlined in this section are merely historically-based labels of evidence pools that largely relate to a central system or concept. It is increasingly being recognized that pathologies in dementia patients are most often mixed (J. A. Schneider, Arvanitakis, Bang, & Bennett, 2007), and that the blending of these pathologies has significant impacts on the heterogeneity in AD (discussed in Section 1.1.3). With this in mind, it is important to consider that each of the pathways/mechanisms described below occur simultaneously and interactively within the same individual.

1.2.2 Cholinergic

The cholinergic hypothesis of AD was born from work in the 1960s and 1970s seeking to identify a neurochemical signature of AD, similar to the dopaminergic deficits observed in Parkinsons's disease (Bernheimer, Birkmayer, Hornykiewicz, Jellinger, & Seitelberger, 1973). The hope was that this would to lead to the development of novel pharmacological interventions for AD (Francis, Palmer, Snape, & Wilcock, 1999). Early evidence of substantial loss of choline acetyltransferase (ChAT) (Bowen, Smith, White, & Davison, 1976; P. Davies & Maloney, 1976), the enzyme responsible for synthesizing the neurotransmitter acetylcholine (ACh), was paralleled by studies revealing the important role of acetylcholine in cognition (specifically deleterious effects of scopolamine, an anticholinergic drug, on memory performance (Drachman & Leavitt, 1974) that could be reversed upon treatment with the anticholinesterase physostigmine (Bartus, 1978)). Paired with the established function of neocortical cholinergic neurotransmission in learning and memory (Deutsch, 1971), and observations of a marked loss of cholinergic neurons in the basal forebrain of patients with AD (Whitehouse et al., 1982), this led researchers to hypothesize that the degradation of cholinergic neurons and deficits in cholinergic neurotransmission were significant contributors to cognitive deterioration in AD.

Studies in the late 1980s and early 1990s examined all aspect of cholinergic neurotransmission in relation to the risk for AD and progression of symptoms, finding evidence both for and against

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the specificity for and necessity of cholinergic deficits in AD; these studies can mostly be grouped as those investigating ACh levels in brain tissue (either directly or by measuring ChAT activity), muscarinic cholinergic receptor density, and lesion studies of cholinergic neurocircuitry in animal models (Contestabile, 2011). While early studies showed that ChAT activity was consistently lower in AD patients vs. age-matched controls, other studies focusing on muscarinic acetylcholine receptor density converged to suggest that there were no AD-specific effects, only those associated with normal aging (Bartus, Dean, Beer, & Lippa, 1982). Contradicting the role of cholinergic system deficits in AD pathogenesis, it has also been shown that the number of cholinergic neurons in the nucleus basalis of Meynert is not different between cognitively normal and MCI patients (Gilmor et al., 1999) and that ChAT activity is actually increased in the frontal cortex and hippocampus of MCI patients (DeKosky et al., 2002).

Despite contradictory findings in the field, the moderate success of acetylcholinesterase inhibitors (AChEIs) in alleviating memory deficits in trangenic animal models (Benzi & Moretti, 1998) led to the development of cholinesterase inhibitor drugs for the treatment of AD symptoms in humans (discussed in Section 1.5). The relative ineffectiveness of AChEIs in reducing risk for or delaying age-at-onset of AD, however, has fueled skepticism over the true relevance of achetylcholine in AD etiology, and many believe that the failure of AChEIs to cure AD has provided definitive evidence against the cholinergic hypothesis. With the emergence of the amyloid cascade hypothesis in the mid-1990s (discussed in the next section) and evidence suggesting that cholinergic deficits may be a secondary result of amyloid toxicity (Roberson & Harrell, 1997), the popularity of the cholinergic hypothesis of AD saw substantial decline and a shift took place in the field toward understanding the interaction of cholinergic activity and amyloid deposition (X. Zhang, 2004).

More recently, multiple revisions have been proposed to the cholinergic hypothesis. Substantial evidence points to ACh as a moderator of the release of brain growth factors (neurotropins), especially the expression of BDNF in the hippocampus (Ferencz et al., 1997; Kokaia et al., 1996). Craig et al. (Craig et al., 2011) emphasize the neuroplastic roles of ACh and suggest that the cholinergic depletion observed in AD may prevent the brain from adequately compensating for subthreshold pathogenic insults and injury such as minor stroke, ischemia, and epileptiform activity. The role of ACh as an age-related moderator of neuronal plasticity is attractive as it supports theories of brain susceptibility to AD-related pathogenesis that align with its depletion

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in AD and the increase in AD risk associated with age -- the so-called "co-factor" theory (R. J. McDonald, 2002).

In summary, it is now accepted that cholinergic deficits, while characteristic of late stage AD and important to the neurobiological processes underlying its cognitive symptoms, are secondary to etiopathogenic factors that have yet to be understood. ACh may play an important role in modulating neuroplastic compensation for AD pathology.

1.2.3 Amyloidogenic

The Amyloid cascade hypothesis was first proposed by John Hardy in 1992 (Hardy & Higgins, 1992). This hypothesis stemmed from seminal work in the 1980s finding that the "senile" plaques defining AD pathology were made of Aβ peptide and that genetic linkage studies of FAD pointing to chromosome 21 were in fact tagging mutations in the Aβ precursor protein gene (*APP*; chr21q21.3). Further evidence came from Levy et al. (Levy et al., 1990), who found that mutation in *APP* could cause cerebral hemorrhage with amyloidosis (Dutch type), demonstrating that APP changes could directly result in cerebrovascular amyloid deposition.

The hypothesis states that neurodegeneration in AD is caused by accumulation and aggregation of the Aβ peptide, which forms the plaques required for its diagnosis. This accumulation can occur either via overproduction of Aβ resulting from aberrant APP processing or via deficiencies in the clearance of Aβ from the brain. This imbalance of Aβ production and clearance, according to the original hypothesis, is the driver of all other pathologies seen in AD, including neurofibrillary tangle deposition, chronic inflammation, and cerebrovascular disease (Hardy & Higgins, 1992; Hardy & Selkoe, 2002).

The processing of APP can be either non-amyloidogenic (does not produce Aβ) or amyloidogenic (produces Aβ) (reviewed in detail by Wilquet and De Strooper (Wilquet & Strooper, 2004)). In non-amyloidogenic processing, newly synthesized APP is quickly transported to the neuronal surface at synaptic terminals (Koo et al., 1990) and is proteolyzed by α-secretase to form soluble sAPPα, which precludes further potentially pathogenic processing. In amyloidogenic processing, APP is internalized into endosomes (Kinoshita et al., 2003) and

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cleaved by β-secretase (BACE1) and γ-secretase to form Aβ. Different forms of Aβ (discussed below) tend to aggregate and form insoluble amyloid fibrils that then form plaques. An active area of research is concerned with understanding why some APP is processed at the cell surface and why some is internalized; the retromer complex of molecules (with which the sortilin-like receptor (SORL1) is associated), responsible for the recycling of APP from endosomes to the cell surface via the trans-golgi network, may play an important role (Q.-Y. Zhang, Tan, Yu, & Tan, 2015).

The forms of Aβ most studied in relevance to AD pathology are the 40- and 42-amino acid length forms ($A\beta_{40}$ and $A\beta_{42}$, respectively), both produced by γ-secretases. $A\beta_{40}$ accounts for approximately 90% of all Aβ released from neurons and increases significantly only in the late stages of AD. In contrast, $A\beta_{42}$ accounts for 10% of secreted Aβ, is found in the early stages of disease, and is found in greatest concentrations in the neuritic plaques associated with AD (likely due to their higher propensity for aggregating into insoluble fibrils) (Citron et al., 1996; Roher et al., 1993). Neuritic plaques (also called "senile", "dense core", or "mature") are large extracellular fibrous deposits seen by microscope when stained using any number of immunohistochemical agents. They are made of dystrophic neurites (that is, remnants of neurons that have lysed), active microglia (the brain's resident immune cells, discussed in Section 1.2.6), and a dense $A\beta_{42}$-rich core. While these neuritic plaques are required for the postmortem diagnosis of AD (along with NFTs), they are not the only type of Aβ plaque. Diffuse amyloid plaques (also "burned-out", "immature", or "cotton wool") are in fact the most abundant form of amyloid deposit found in the brain (Yamaguchi, Hirai, Morimatsu, Shoji, & Harigaya, 1988). However, diffuse plaques are not considered in the diagnosis of AD, as they do not include dystrophic neurites or accumulations of glial cells, and do not appear to have pathological effects on neurons situated within them (H. Braak & Braak, 1991; D'Andrea & Nagele, 2010). Once thought to be only a stage of neuritic plaque formation, diffuse plaques are often found in healthy aging and have been proposed to signal a shift in the amyloid cascade from pathological to non-pathological amyloid deposition. Finally, even though extracellular neuritic plaques have clear association with AD progression and are required for its diagnosis, it seems the small soluble oligomeric forms of Aβ (2-6 peptides in length, found pooled within neuritic plaques), and intracellular $A\beta_{42}$ (LaFerla, Green, & Oddo, 2007) are likely the true pathogenic factors (Walsh et al., 2002).

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Following the initial description of the amyloid cascade hypothesis, a number of key pieces of evidence emerged supporting it (outlined by Hardy and Selkoe (Hardy & Selkoe, 2002)). First, mutations in the genes encoding for the gamma secretase enzyme subunits responsible for processing APP (*PSEN1* and *PSEN2*) were shown to cause FAD (Levy-Lahad et al., 1995; Rogaev et al., 1995; Sherrington et al., 1995), likely though altered APP metabolism and $A\beta_{42}$ deposition (Karen Duff et al., 1996). Second, mutations in the gene encoding for the tau protein (*MAPT*; chr17q21.1) were found to cause the non-amyloidogenic frontotemporal dementia with parkinsonism (Hutton et al., 1998), suggesting that severe pathological alterations in tau (resulting in NFT deposition and neurodegenration) are not sufficient to generate amyloid plaques found in AD. Third, transgenic mice expressing mutant forms of both APP and tau show increased NFT deposition compared to those only expressing mutant tau (J. Lewis et al., 2001), suggesting that Aβ is necessary for and precedes NFT-related neurotoxicity. Fourth, transgenic mice overexpressing mutant APP showed reductions in Aβ deposition when crossed with mice deficient for APOE (Bales et al., 1997), suggesting that the unequivocal *APOE* genetic risk factor for AD possibly conferred such risk through alteration of Aβ metabolism. Fifth, early genetic association and quantitative trait loci (QTL) studies scanning the genome for regions in which mutation influences risk for late-onset AD identified regions other than chromosome 21 that were also associated with $A\beta_{42}$ levels (Kehoe et al., 1999; Myers et al., 2000), and Aβ neurotoxicity has been demonstrated in cultured neurons (Whalen, Selkoe, & Hartley, 2005) and mouse models (Shankar et al., 2008). Finally, preliminary results of the Biogen-sponsored (Cambridge, MA, USA) PRIME trial clinical (NCT01677572) of the experimental anti-amyloid drug Aducanumab (BIIB037) in Aβ-positive early AD subjects showed improvement of cognition in some subjects. Even more recently, the Eli Lilly and Company-sponsored (Indianapolis, IN, USA) EXPEDITION–EXT clinical trial (NCT01127633), an extension of the failed solenazumab trials, demonstrated a modest disease-modifying effect in mild AD (Karran, 2015).

Despite the evidence in favour, a number of important contradictory observations have been made that dispute the amyloid cascade hypothesis. Karl Herrup recently provided a critical review of the hypothesis, summarizing weaknesses exposed from genetic, biochemical, animal model, pathological, clinical, and epidemiological lines of inquiry (Herrup, 2015). First, no mutations in α-secretase have been found to influence risk for FAD. Second, mouse models

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expressing only Aβ do not develop dementia-like symptoms and the perturbation of biochemical pathways other than amyloidogenic (inflammatory, vascular) are sufficient to induce dementia (Webster, Bachstetter, Nelson, Schmitt, & Van Eldik, 2014). Third, AD mouse models do not show appreciable neurodegeneration. Treatments often induce full reversal of quick-onset cognitive and behavioral changes, which demonstrates the inability of transgenic mice to accurately model human AD (Dodart et al., 2002; J. Xu et al., 2014). Fourth, NFTs show better correlation with neurodegeneration than plaques (discussed in Section 1.2.4) and many cognitively normal elderly are found to show significant Aβ pathology before (Villemagne et al., 2011) and after death (Davis, Schmitt, Wekstein, & Markesbery, 1999). Finally, results from clinical trials showing the ineffectiveness of Aβ clearance after symptom onset, the failure of aforementioned phase 3 anti-amyloid trials, and the partial successes of interventions acting on supposed downstream processes all point toward amyloid as a secondary, rather than causal pathogenic event. Herrup also cautions that the field's reliance on the amyloid cascade hypothesis has resulted in circular logic that may hamper progress whereby the definition of disease depends on plaque deposition (i.e. amyloid positivity without dementia is thought to represent "preclinical AD", rather than a potentially distinct condition).

Nonetheless, a majority of scientists in the field are compelled by the evidence for involvement of Aβ as an important contributor to the risk for and progression of AD, though most now acknowledge the complexities of the underlying causal pathways that lead to AD and understand that a more nuanced model is required. Accordingly, several revisions to the amyloid cascade hypothesis have been proposed. Karran et al. (Karran, Mercken, & De Strooper, 2011) outline possible mutually exclusive roles of Aβ in AD: those as a "trigger", "threshold", or "driver" of resultant tau protein-related pathology and neurodegeneration. While accumulating evidence (discussed above) seems to reject the "trigger" role of Aβ in AD pathogenesis (which is a central tenet of the original amyloid cascade hypothesis), the possibility that various Aβ species function either at threshold or continuously to drive AD pathology remains viable. Alternatively, McGeer and McGeer (McGeer & McGeer, 2013) have proposed an "amyloid cascade-inflammatory hypothesis" of AD etiopathology that emphasizes the importance of the Aβ-driven inflammatory response (discussed in Section 1.2.6). This demonstrates the growing trend toward viewing pathogenic mechanisms as concurrent and interdependent, rather than isolated.

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Further revision of the amyloid cascade hypothesis is necessitated by studies examining normal physiological roles of amyloid. The model that more Aβ is bad and less is good does not accurately capture the current state of knowledge on the non-toxic cellular functions of Aβ and APP (reviewed by Atwood et al. (Atwood et al., 2003)). Evidence that conditions of ischemia (Jendroska et al., 1995), hypoglycemia (Shi, Xiang, & Simpkins, 1997), and traumatic brain injury (Murakami et al., 1998) can induce the overexpression of APP and a shift from non-amyloidogenic to amyloidogenic processing support a protective role of amyloid in disease. APP has been shown to modulate neurotrophic signaling (Hasebe et al., 2013) and is necessary for the maintenance of neuronal integrity in hippocampus of mice (Tyan et al., 2012). Despite the ability of Aβ peptide to induce oxidative stress, confusingly it also has anti-oxidant properties thought to be a response to intracellular increases in transition metals (in particular copper) that catalyze the generation of reactive oxygen species (ROS) (Opazo et al., 2002; Sinha, Bhowmick, Banerjee, & Chakrabarti, 2013). The structure of the $A\beta_{42}$ peptide makes it an effective chelator of these transition metal ions and thus may act to inhibit the catalysis of damaging ROS (Atwood et al., 2000). However, the aggregation of Aβ ablates its oxidative functions and results in toxicity to mitochondria via mechanisms that are independent of effects on ROS (e.g. membrane depolarization). An explanatory model proposed by Anatol Kontush (Kontush, 2001) suggests that the early protective amyloid response generates Aβ-metal complexes as ROS-generating ions are chelated. This leads to the production of both highly-toxic Aβ oligomers and non-toxic diffuse amyloid plaques, which are inversely correlated with oxidative damage (Nunomura et al., 2000) and may function to encapsulate and deactivate Aβ oligomers (Cuajungco et al., 2000). As the underlying disease processes progresses, the production of Aβ oligomers overcomes the counteractive detoxifying effects of diffuse plaques, neurons degenerate, neuritic plaques begin to form, and cognitive decline begins.

In summary, the amyloid cascade hypothesis has provided some of the most compelling and actionable evidence for AD etiopathogenesis to date. However, recent studies have called into question the causal role of Aβ in AD, instead postulating that its accumulation may be a secondary effect of earlier causal events underlying the disease. Depending on the stage of amyloid deposition, Aβ peptides may exert beneficial or detrimental effects on cellular viability.

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1.2.4 Tauopathic

In 1986 it was discovered that the NFTs found in AD brain were composed of the microtubule-associated protein tau, which was organized in polypeptides to form paired helical filaments (PHFtau) (Grundke-Iqbal et al., 1986). The importance of tau dysfunction in neurodegeneration was demonstrated following the discovery that mutations in the gene encoding tau (*MAPT*) could cause frontotemporal dementia with parkinsonism (Hutton et al., 1998) – as mentioned previously this was taken by some as evidence supporting the amyloid cascade hypothesis, due to lack of amyloid deposition in this disease (Section 1.2.3).

The normal function of tau in the brain is promoting the assembly of tubulin and maintaining the stability of resulting microtubules, which act as essential structural scaffolds and intracellular transport networks (Drubin & Kirschner, 1986). When tau is hyperphosphorylated (by any of several kinases including glycogen synthase kinase-3 (GSK3), cyclin-dependent protein kinase-5 (cdk5), and mitogen activated protein ERK 1/2 (T. J. Singh, Grundke-Iqbal, McDonald, & Iqbal, 1994)), it fails to interact with tubulin, thereby destabilizing microtubules (Alonso, Zaidi, Grundke-Iqbal, & Iqbal, 1994). This hyperphosphorylated tau is thought to be responsible for the breakdown of microtubules (which are more abundant in neurons than any other tissue) and subsequently aggregates into PHFtau and NFTs (Köpke et al., 1993; Vincent, Zheng, Dickson, Kress, & Davies, 1998), though some evidence shows that microtubule number and length are reduced in AD compared to controls in a manner that is not correlated with PHFtau (Cash et al., 2003). Once the NFT has elicited cytotoxic effects on its host neuron (mechanisms include impaired axonal transport, DNA damage, chromatin remodeling, mitochondrial dysfunction, and aberrant cell cycle activation (Frost, Götz, & Feany, 2015)), the neuron degrades, lyses, and releases it into extracellular space, resulting in a so-called “ghost tangle”, which is considered highly indicative of neurodegeneration (F. Braak, Braak, & Mandelkow, 1994).

In 1991, Heiko and Eva Braak used advanced silver staining techniques in the brains of 83 demented and non-demented subjects to describe the topological and temporal progression of AD pathology, identifying distinct stages of both amyloid and NFT deposition corresponding loosely to the relative cognitive progression of the illness (H. Braak & Braak, 1991). Importantly, they noticed that the presence of NFTs generally preceded that of neuritic plaques and that, in contrast to neuritic plaques which were often present in non-demented brains and

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showed inconsistent distributions and densities, NFTs had a well-defined pattern that allowed them to identify six stages of pathological progression correlating strongly with disease status and loss of neurons in gray matter (commonly referred to as "Braak stages"). The stages include "transentorhinal" (I/II; usually clinically silent), "limbic" (III/IV; early AD or MCI), and "isocortical" (V/VI; AD dementia), where roman numerals indicate both early and late sub-stages (six total).

The very early detection of NFTs and staging of AD well before symptoms emerge have drawn attention to the lack of understanding of what differentiates healthy aging with AD-related neuropathology from AD itself. The instability of the earliest phases of AD (i.e. the tendency for individuals with MCI to not progress to AD or even revert to normal (Manly et al., 2008)) add uncertainty to the meaning of NFT-specific pathology.

In summary, strong evidence supports the idea that tau-related pathology precedes Aβ-related pathology; however, it is clear that both are required to cause the cognitive deficits and neurodegeneration seen in AD. Further, despite the causal role of amyloid in FAD and of tau in FTD with parkinsonism-17 (FTDP-17), the presence of either and/or both are only risk factors for late onset AD and some evidence suggests that microtubule changes may even precede the hyperphosphorylation of tau.

1.2.5 Vascular

For most of the 19th century, long before the introduction of the term Alzheimer's disease, it was thought that arteriosclerosis – the narrowing and hardening of blood vessels - was the primary driver of brain dysfunction associated with senile dementia (Beach, 1987; Loeb, 1995). In fact, the brain of patient Auguste D was noted as having arteriosclerotic changes in addition to plaque and tangle pathology (Alzheimer et al., 1995). In 1955, Martin Roth published a study examining hospital records for 464 patients with different forms of psychosis; patients included those with "senile psychosis", similar to a modern description of AD, and "arteriosclerotic psychosis", which denotes more focal, fluctuating symptoms likely due to cerebrovascular disease, and characterized by "emotional incontinence, the preservation of insight, or epileptiform seizures" (Roth, 1955). Roth noticed significant differences in short-term (6-month) outcomes between the

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arteriosclerotic and senile psychosis patients that were not due to age, providing the first indirect evidence for a clinical distinction between AD and a vascular dementia (VaD). In an attempt to clarify the difference between AD symptoms resultant from vascular changes and those as a result of a non-AD dementia, the National Institutes of Health/National Institute of Neurological Disorders and Stroke (NIH/NINDS) guidelines currently make the distinction between vascular cognitive impairment (VCI) (Moorhouse & Rockwood, 2008) and vascular dementia (VaD). This classification unfortunately suffers from some of the same circular logic plaguing the definition of pre-clinical AD (i.e. the presence of pathology in cognitively normal individuals defining a pre-emergent form of AD).

The recognition of cerebrovascular pathology in patients with AD (Vermeer et al., 2003), combined with evidence from epidemiological studies finding common risk factors for vascular disease and AD (e.g. diabetes mellitus, midlife hypertension, and hyperlipidemia; outlined in Section 1.1.5) (Luchsinger et al., 2005), has led to two hypotheses: **1)** vascular changes generate risk for AD neuropathology and hence AD diagnosis, and **2)** vascular changes generate risk for parallel cerebrovascular pathology that contribute to the likelihood of dementia.

The etiopathogenic vascular hypothesis of AD **(hypothesis 1)** essentially states that cerebrovascular changes, including stiffening of blood vessels, cause restriction of cerebral blood flow and hypoperfusion. This hypoperfusion is thought to be the driver of secondary energy crises that lead to other pathological cascades. Strong proponents of this hypothesis (notably including Juan-Carlos de la Torre) assert that vascular deficits can fundamentally explain all facets of AD pathology, whereby microcirculatory disturbance of oxygen and glucose delivery to brain tissue causes early ischemic changes in oxidative phosphorylation and ATP generation that lead to an immune response (discussed in Section 1.2.6) and reactive overexpression of APP, leading to plaque pathology, cytoskeletal damage and NFT formation (de la Torre, 2002, 2002, 2004, 2012; de la Torre & Mussivand, 1993).

Others have contested the etiopathogenic role of vascular irregularities in AD, citing critical literature suggesting that oxidative stress precedes pathogenic changes in cerebrovascular endothelium and resulting inflammation. It has been shown that amyloid-related cerebrovascular dysfunction in transgenic mice can be rescued by the application or up-regulation of superoxide dismutase (a potent free radical scavenger) (Iadecola et al., 1999). This is consistent with

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evidence that mitochondrial dysfunction is a primary driver of early AD pathogenesis (an old and enduring concept (Quastel, 1932; Swerdlow & Khan, 2009)), including observed dysregulation of mitochondrial fission and fusion proteins in AD (Xinglong Wang et al., 2009), altered glucose metabolism in early AD (Minoshima et al., 1997), and abnormalities in critical mitochondrial enzymes in young *APOE* ε4 carriers (Valla et al., 2010) and elderly MCI subjects (Chandrasekaran et al., 1994). Further, it is not clear if arteriosclerotic damage can lead to cognition-impacting ischemia in AD in the absence of more developed lesions, such as infarcts (Bangen et al., 2015).

The idea that vascular pathology (driven either by early vascular changes or other AD pathological cascades (i.e. pre-tangle hyperphosphorylated tau, oligomeric Aβ, or mitochondrial dysfunction) additively influences risk for cognitive deficits and dementia when present alongside AD-related pathology **(hypothesis 2)** has gained traction in recent years. Bangen et al. (Bangen et al., 2015) conducted an autopsy study of 602 subjects ages 36-104, comparing antemortem vascular risk with postmortem cerebrovascular changes (including lacunar infarcts, microinfarcts, arteriosclerosis, and atherosclerosis in the circle of Willis), AD-related neuropathology, and cognition. They found that antemortem vascular risk score was predictive of postmortem cerebrovascular changes, but not AD severity or AD-related amyloid deposition (including amyloid deposited around blood vessels, known as cerebral amyloid angiopathy (CAA)). This study highlights three major challenges of assessing the debate over the etiopathogenic vs. concomitant role of vascular pathology in AD, which is still unresolved (Chui, Zheng, Reed, Vinters, & Mack, 2012). First, undetected cerebrovascular pathology (such as silent brain infarcts) may cause symptoms labelled as AD – most cases of probable AD are found to have comorbid vascular pathology postmortem (J. A. Schneider et al., 2007; J. A. Schneider, Arvanitakis, Leurgans, & Bennett, 2009). Second, autopsy studies of convenience samples likely have intrinsic selection biases including reasons for the subjects seeking medical attention, reasons for autopsy consent, and restrictive inclusion criteria (Chui et al., 2012). Third, the link between AD-related (i.e. Aβ and NFT) and vascular pathology is complex and not easily modeled; it has been shown in mouse models that an increased ratio of $A\beta_{40}$ to $A\beta_{42}$ may determine a preference for amyloid plaques to form around vasculature (CAA or parenchymal amyloid (possibly due to differential extracellular movement of Aβ species) (Herzig et al., 2004),

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and CAA may interact with other non-vascular-specific AD pathology to influence cognitive deficits in AD (Pfeifer, White, Ross, Petrovitch, & Launer, 2002).

In summary, cerebrovascular pathologies such as arteriosclerosis and cerebral infarcts are present in AD cases to a greater degree than in age-matched controls and contribute to symptom severity. Whether these pathologies cause or occur in parallel to AD pathology is unclear; vascular changes associated with AD are difficult to disentangle from neuroinflammatory processes, as they modulate and exert neurodegenerative effects via each other.

1.2.6 Inflammatory

The term "inflammatory hypothesis of AD" is quite new, first coined by Dimitrije Krstic and Irene Knuesel in 2013 (Krstic & Knuesel, 2013); however, Sheng et al. (Sheng et al., 1996) first proposed that an inflammatory cytokine (IL-1) may be a driver of AD pathogenesis in 1996. Despite the recent attention it has received, neuroinflammation is as much a hallmark of AD pathology as Aβ plaques and NFTs and was described in Alzheimer's initial case report in 1907 (Alzheimer et al., 1995). As noted in Section 1.2.3, neuritic plaques are composed of a dense Aβ core surrounded by dystrophic neurites and active microglia, the brain's resident immune cells (Akiyama et al., 1999; Mackenzie, Hao, & Munoz, 1995). The immune response in the brain is thought to be mediated by these microglial cells (Giulian, 1987). In 1988, it was shown that the class II major histocompatibility complex (MHC) antigen HLA-DR could be used to identify microglial reactivity in brain tissue, allowing for probing of inflammatory mechanisms in postmortem AD (Rogers, Luber-Narod, Styren, & Civin, 1988). It has since been shown both at autopsy and *in vivo*, that inflammatory processes are more active in the brains of AD and MCI patients than in healthy controls (Hommet et al., 2014). The root cause of neuroinflammation in AD, however, is not known; evidence shows that the process can be initiated by damaged neurons, Aβ deposits, and/or NFTs (Akiyama et al., 2000).

Perhaps not surprisingly, some of the most compelling evidence for a causal role of inflammation in AD came from the originators of the hypothesis and led to its formation; in 2012, Krstic et al. (Krstic et al., 2012) demonstrated that a single systemic immune system disturbance (by injection of a double-strand RNA that mimics a virus, called PolyI:C) during late

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gestation of a wild-type mouse was sufficient to induce AD-like neuropathological changes and cognitive decline (specifically spatial recognition memory, as measured by the Y-maze task). Mice that sustained two immune challenges (one during gestation, the other during adulthood (between 9-15 months)) showed even more pronounced AD-like changes, including APP deposition (in entorhinal regions), PHFtau aggregation, microglial activation, and reactive gliosis. In humans, it has been found that infection burden (including cytomegalovirus, herpes simplex virus type 1 (HSV-1), and bacteria) increase odds for developing AD (Bu et al., 2014).

Further support for the inflammatory hypothesis of AD has come from genome-wide association studies (GWAS) that have consistently identified variants within genes involved in inflammation as risk factors for AD, albeit with modest effects (Karch et al., 2014; Lambert et al., 2013; Naj et al., 2011). A recent pathway analysis conducted by the International Genomics of Alzheimer's Disease Consortium (IGAP) on data from their meta-analysis of over 74 000 individuals showed the most significant enrichment for genes involved in the immune response (L. Jones et al., 2015). Additional evidence comes from studies finding that mutations within the gene encoding for triggering receptor expressed on myeloid cells 2 (TREM2) are risk factors for AD (Guerreiro et al., 2013; Jonsson et al., 2013). TREM2 is a cell membrane receptor involved in signaling pathways leading to activation of immune cells, including microglia. Subsequent *in vivo* and *in vitro* studies of human and mouse tissue have demonstrated that dysfunctional TREM2 leads to dysregulation of the immune response, though whether the protein functions to compensate for AD-related pathology (Jiang et al., 2014) or exacerbate it (Jay et al., 2015) is not clear.

As discussed in Section 1.2.5, the immune response to pathogenic vascular changes is a well-documented phenomenon, but it has also been shown that aberrant activation of the immune system can create vascular deficiencies and leakage of the blood-brain barrier (BBB), making their temporal relationship unclear (S. L. Lim, Rodriguez-Ortiz, & Kitazawa, 2015). Temporal relationships of inflammation with other AD pathologies are also unclear: in transgenic mice, the levels of both $A\beta_{40}$ and $A\beta_{42}$ expressed were related to increases in pro-inflammatory cytokines (including interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα), interleukin-6 (IL-6), interleukin-1 beta (IL-1β), interleukin-1 alpha (IL-1α), and granulocyte macrophage colony-stimulating factor (GM-CSF)) (N. S. Patel et al., 2005), though changes in immune system reactivity also occur with normal aging (Teunissen et al., 2003).

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Importantly, it is not currently understood if the activation of microglia is a beneficial or detrimental process. Evidence shows that active microglia facilitate the clearance of Aβ (Mandrekar-Colucci & Landreth, 2010), however, the release of pro-inflammatory molecules such as IL-1β, IL-6, and TNFα can have toxic effects (intended to destroy invading pathogens). Fueling this complexity is the fact that microglia may exhibit very different characteristics depending on their state of activation, which can be "classical", "alternative" (also called "repair/resolution"), or "acquired deactivation" (Colton, 2009); the first falling into the *M1-like* category of microglial phenotypes (pro-inflammatory/destructive) and the second two falling into the *M2-like* (anti-inflammatory/protective) category (an oft-reported, simplified nomenclature developed to helpfully decipher opposing microglial phenotypes (Y. Tang & Le, 2015)). Gene expression profiling studies in transgenic mouse models show that microglial phenotypes in AD are likely in a repair-primed alternative state of activation (Colton et al., 2006). Consistent with the protective roles of microglia in AD are early observations of dystrophic and apoptotic microglial in AD brain (Lassmann et al., 1995; Streit, 2002). The change of activation from M2- to M1-type as the immune response persists in reaction to the unrelenting accumulation of Aβ and/or tau pathology may represent a key turning point in AD pathogenesis (Hanisch & Kettenmann, 2007). However, despite known destructive properties of pro-inflammatory cytokines, it has been shown that anti-inflammatory signaling factors can also have detrimental effects on bran immune function and exacerbate AD-like symptoms (Chakrabarty et al., 2012; Town et al., 2008).

In summary, AD is characterized by widespread neuroinflammation involving the activation of resident immune cells (microglia) and astrocytosis. This activation may be a protective response to invading pathogens or a destructively aberrant process causing neurodegeneration, or both simultaneuously. While AD etiopathogenesis certainly involves neuroinflammation, a clear chain of causality including the other major AD pathogenic mechanisms has not been delineated.

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1.3 Alzheimer's Disease Biomarkers

1.3.1 Background

Given the growing appreciation for AD as a lifelong process, with clinical signs and symptoms only emerging late in the disease, and the failure of clinical trials for interventions targeting symptomatic patients, there is now a critical emphasis on the use of "biomarkers" in AD research to detect and define early stage disease. Broadly, a biomarker is any medical sign (i.e. an objective measure of a medical state independent of the patient's own perception) that can be measured accurately and reproducibly (Strimbu & Tavel, 2010).

In the 1984 NINCDS-ARDA Work Group diagnostic criteria (McKhann et al., 1984), a number of "laboratory assessments" (essentially biomarkers) were identified that could improve the diagnostic accuracy by eliminating other causes of dementia. These included electrophysiology (e.g. electroencephalography (EEG); sensitive but not specific), computed tomography (CT), regional cerebral blood flow (e.g. xenon clearance; may differentiate between some dementias), PET imaging (amyloid imaging had not yet been invented, but glucose and oxygen uptake were commonly measured), MRI (identifying demyelination and white matter hyperintensitites), and examination of body fluids and non-neural tissues (including cerebrospinal fluid (CSF) and blood). When the criteria were revised in 2011 (McKhann et al., 2011), they took into account over two decades of biomarker research (Hampel et al., 2008) implicating three specific modalities that could be incorporated into a diagnosis of "probable AD dementia with evidence of the AD pathophysiological process": CSF $A\beta_{42}$, tau, and hyperphosphorylated tau (P-tau) levels, PET imaging amyloid uptake in temporo-parietal cortex, and structural MRI analysis of disproportionate atrophy in temporo-parietal regions. Clifford Jack and David Holtzman group these biomarkers into two major categories: amyloidogenic and neurodegenerative (Clifford R. Jack & Holtzman, 2013).

Based on changes in such biomarkers, Sperling et al. (Sperling et al., 2011) proposed a three-stage system for tracking preclinical AD leading to MCI; the stages are 1) asymptomatic cerebral amyloidosis, 2) amyloid positivity + evidence of synaptic dysfunction and/or early neurodegeneration, and 3) amyloid positivity + evidence of neurodegeneration + subtle cognitive decline. While these stages, which were based primarily on biomarker trajectories proposed by Jack et al. (Clifford R. Jack et al., 2010), do not constitute clinical diagnoses, they were intended

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to aid in the design of future studies aimed at understanding the AD disease process and of clinical trials targeting at-risk individuals who have not yet entered the later stages of disease.

In this thesis, I have focused on the use of molecular and neuroimaging endophenotypes (or "intermediate" phenotypes) -- heritable characteristics that co-segregate with the illness in question, are state independent (i.e. remain detectable even in the absence of outward symptoms), and are found in family members without the disease (Gottesman & Gould, 2003) -- that have been validated as predictive of AD risk by previous studies to unearth the temporo-spatial patterns of disease risk conferred by common genetic variants. Biomarkers used in the work presented here include plasma and serum proteomics, *in vivo* molecular neuroimaging (amyloid PET), and *in vivo* structural and diffusion MRI. Postmortem measurements of AD-related pathologies (amyloid, tau, and microglial activation), as discussed in Section 1.2, have been included in Chapters 4, 5 and 6, in attempts to confirm and extend the mechanistic interpretations of *in vivo* biomarkers used in those studies, but will not be discussed further here. However, cerebrospinal fluid proteomics, while not included in studies presented herein, will be discussed because of its current use in AD diagnostics.

1.3.2 Cerebrospinal Fluid Biomarkers

The analysis of CSF from living subjects offers a window into the molecular contents of the central nervous system (CNS), as it is in direct contact with the extracellular space of the brain. Levels of core CSF biomarkers ($A\beta_{42}$, total tau (T-tau), and P-tau) have been consistently shown to differentiate between AD, MCI, and CN subjects, whereby the ratio of $A\beta_{42}$/T-tau and $A\beta_{42}$/P-tau decrease with worsening disease (reviewed by Blennow et al. (Blennow, Hampel, Weiner, & Zetterberg, 2010) and Sonnen et al. (Sonnen, Montine, Quinn, Breitner, & Montine, 2010)). The sensitivity for diagnosis of AD based on CSF $A\beta_{42}$ and T-tau is approximately 78-84% when considered independently, but up to 86% when both are combined (with specificity improving from 84-90% to 97%) (Maddalena et al., 2003). Based on the effectiveness of this combination, the INNOTEST® (Fujirebio Europe N.V., Ghent, Belgium) Amyloid Tau Index (IATI; calculated as IATI = $A\beta_{42}$/(240+1.18*T-tau)) is a commercially available enzyme-linked immunosorbent assay (ELISA), which has demonstrated potential use in a clinical setting (Vanderstichele et al., 2006).

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Since AD is largely a diagnosis of exclusion, a priority in the field has been identifying CSF biomarkers that are capable of differentiating between AD and other types of clinically similar dementias (including FTD, VaD, and dementia with Lewy bodies (DLB)). Some groups have shown that the ratio of $A\beta_{42}$ to tau hyperphosyphorylated at threonine 181 ($P\text{-}tau_{181P}$) outperforms $A\beta_{42}$/T-tau ratio and either $A\beta_{42}$ or T-tau alone in classifying postmortem confirmed AD from FTD and DLB subjects (Struyfs et al., 2015). Different hyperphosphorylated species of tau, including $P\text{-}tau_{231P}$ and $P\text{-}tau_{199P}$, may provide heightened specificity and sensitivity for the classification of AD vs. FTD, DLB, and VaD (Hampel et al., 2004). However, others have shown that $A\beta_{42}$ performs better than T-tau and P-tau at differentiating AD from other dementias (Ewers et al., 2015).

Much work is currently being directed at identifying CSF biomarker panels that add confidence to the accuracy of diagnosis classification and prediction models. Candidate biomarkers that have shown some promise for improvement of diagnostic sensitivity and specificity are enzymes (including β-site APP-cleaving enzyme 1 (BACE1)), APP isoforms (e.g. sAPPα and sAPPβ), Aβ peptide isoforms (e.g. $A\beta_{14}$, $A\beta_{15}$ and $A\beta_{16}$), Aβ oligomers, endogenous Aβ antibodies, neuronal and synaptic markers of degeneration (e.g. visinin-like protein 1 (VLP-1), neurofilaments, and growth-associated protein 43 (GAP-43)), and markers of oxidative damage (e.g. F2-isoprostanes) (Blennow et al., 2010). Most recently, markers of brain iron load (ferritin) and glutamatergic function (D-serine) have shown promise in predicting conversion from MCI to AD and improving existing diagnostic accuracy of AD (Ayton, Faux, Bush, & Alzheimer's Disease Neuroimaging Initiative, 2015; Madeira et al., 2015). In fact, the combination of IATI with D-serine showed a sensitivity and specificity of 96.3% and 100%, respectively (albeit in a small sample of 17 neuropathologically-confirmed AD and 12 non-AD subjects) (Madeira et al., 2015).

In summary, CSF $A\beta_{42}$, T-tau, and P-tau show robust relationships with AD onset and decline. These core biomarkers also have the ability to differentiate between AD and other forms of dementia, though there is active debate over which markers are most effective.

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1.3.3 Blood-Based Biomarkers

As blood draw is a minimally-invasive and inexpensive procedure, proteomic analysis of circulating plasma (the liquid medium of blood in which blood cells are suspended) or serum (plasma without clotting factors) is an attractive source of biomarkers in AD (Issaq, Xiao, & Veenstra, 2007). Measuring concentrations of proteins in blood to gain insight into brain-specific phenomena has some inherent difficulties, including the contributions of non-brain organs and tissues to the molecular make-up of blood and BBB. While the BBB functions to restrict the movement of substances to and from the brain, the normal absorbance of CSF into blood results in the exchange of some small peptides (S. Patel, Shah, Coleman, & Sabbagh, 2011), and "leakage" of a compromised BBB, as observed in AD, may further lead to the transfer of brain proteins into peripheral circulation (Zipser et al., 2007).

As with CSF, evidence for the involvement of core biomarkers in risk for and progression of AD is abundant, though not as consistent. A meta-analysis by Song et al. (F. Song et al., 2011) on plasma Aβ literature from 1989-2010 found that plasma $A\beta_{42}$ had predictive value in healthy individuals for the development of AD (higher $A\beta_{42}$ = higher risk). However, in cross-sectional studies, $A\beta_{42}$ showed only a trend toward being lower in AD patients vs. healthy controls (p=0.08), and $A\beta_{40}$ showed no difference. Another meta-analysis (Koyama et al., 2012) of 13 prospective plasma biomarker studies published from 1995-2011 found that neither $A\beta_{42}$ nor $A\beta_{40}$ was associated independently with the development of AD, but that the ratio of $A\beta_{42}$: $A\beta_{40}$ was significantly so. In contrast to peripheral Aβ, which appears to indicate early risk for AD, peripheral tau protein levels, as measured by assays hypersensitive to all tau isoforms, are elevated in AD patients compared to both MCI subjects and normal controls, suggesting it may be a late-stage biomarker of disease progression (Zetterberg et al., 2013). However, due to substantial overlap in variance between groups and high heterogeneity between studies, both Aβ and tau blood-based biomarkers lack the discriminatory capacity required for diagnostic use. Importantly, estimates of heritability for plasma $A\beta_{42}$ have been as high as 73% (Ertekin-Taner et al., 2001), satisfying aforementioned criteria for endophentoype status.

Inflammatory blood-based biomarkers have also been tested extensively for association with AD, MCI, and cognitive decline, but with more consistent results. A meta-analysis of 40 studies by Swardfager et al. (Swardfager et al., 2010) concluded that peripheral pro-inflammatory

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biomarkers (including IL-6, TNFα, IL-1β, IL-12, and IL-18) were consistently increased in AD, whereas anti-inflammatory cytokines IL-4 and IL-10 showed no differences. While they did find evidence for increases in anti-inflammatory transforming growth factor beta (TGF-β) in AD vs. controls, it has been shown that TGF- β may exert pro-inflammatory effects in the presence of IL-6 (Veldhoen, Hocking, Atkins, Locksley, & Stockinger, 2006). A smaller meta-analysis of seven studies by Koyama et al. (Koyama et al., 2013) further found evidence for elevation of C-reactive protein (CRP) and IL-6 in all-cause dementia, however differences were less pronounced in AD specifically, suggesting that results for these analytes may be reflective of heterogeneity in diagnoses rather than an AD-specific process. In general, inflammatory biomarkers may be more reflective of CNS changes than other blood-based biomarkers, since there is continuous cross-talk between the peripheral and central immune systems (Perry, Cunningham, & Holmes, 2007), cytokines can cross the BBB via active transport or passive diffusion (Banks, Kastin, & Broadwell, 1995; Rivest et al., 2000), and activated peripheral immune cells can migrate across the BBB (El Khoury & Luster, 2008).

In addition to targeting a-priori analytes, groups have also attempted to develop panels of blood-based biomarkers that best differentiate between AD, MCI, and control groups cross-sectionally. Though studies repeatedly claim high diagnostic accuracy for certain combinations of analytes, the number and identity of the important analtyes is quite variable. For example, the Texas Alzheimer's Research and Care Consortium (TARCC) used a serum-based panel of 30 analytes to achieve sensitivity and specificity of 88% and 82%, respectively, to distinguish clinical AD from cognitively normal controls (O'Bryant et al., 2011). However, the Australia Imaging Biomarkers and Lifestyle Study of Ageing (AIBL) study achieved 85% and 93% with a different panel of 17 analytes in plasma (Doecke et al., 2012). Further demonstrative of the variability in blood-based biomarker discriminatory analyses, Hu et al. (W. T. Hu et al., 2012) analyzed two independent cohorts from the University of Pennsylvania (Philadelphia, PA, USA) and Washington University (St. Louis, MO, USA), finding that while 23 analytes were predictive of diagnosis in both cohorts, six showed opposite directions of effect. In each of the aforementioned studies, the publicly available Alzheimer's Disease Neuroimaging Initiative (ADNI; described in Section 1.3.8) plasma proteomic dataset was also analyzed, each time with different results.

To tackle the major issue of reproducibility (Galasko & Golde, 2013), the first set of international guidelines were released for use in research of blood-based biomarkers (O'Bryant

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et al., 2015), emphasizing the importance of both controllable (e.g. time of sample collection, subject fasting status, needle size, collection tube types and temperature of storage freezers), and uncontrollable (e.g. non-AD comorbidities, subject activity level and diet, and medications) sources of variation and potential confounding that should be taken into account to harmonize future research and improve reproducibility.

In summary, while the interruption of the BBB may result in "leakage" and peripheral detection of CNS molecular processes, the complex nature of molecular communication between body and brain is not well understood. Literature surrounding changes in circulating levels of Aβ and tau proteins in AD is heterogeneous, though some evidence suggests that Aβ may be an early indicator of risk and tau may be a late marker of neurodegeneration. Inflammatory blood-based biomarkers may offer meaningful insight into neuroinflammatory processes due to molecular communication between the CNS and periphery.

1.3.4 Molecular Imaging (Positron Emissions Tomography)

The ability to measure concentrations of specific molecules directly inside the living brain, rather than relying on more distal measurements of fluid biomarkers, has been made possible by PET. This technology was enabled by seminal research by Irène and Frédéric Joliot-Curie demonstrating that radioactive atoms could be created artificially in a laboratory setting (Joliot & Curie, 1934). Their discovery that positrons (the anti-particle to the electron, with a charge of +1e, first mathematically predicted by Paul Dirac in 1931 (Dirac, 1931) and experimentally confirmed in 1933 (Blackett & Occhialini, 1933)) continued to be emitted from target substances (such as magnesium, boron, or aluminum) after bombardment by alpha particles earned them the Nobel Prize for chemistry in 1935. Simultaneously, a group led by Ernest Lawrence had conceived the cyclotron, a magnetic device capable of generating high-energy particles (protons and deuterons) for the bombardment of other elements. Originally designed to explore the properties of the atomic nucleus, it became a medical device (called the "medical cyclotron") for the production of artificial radioisotopes, including 11C, 13N, 15O, and 18F (Wagner Jr, 1998). This ushered in the era of nuclear medicine, whereby photon-emitting isotopes were used as radiotracers and chemically bound to molecules of known physiological function. Currently, radiotracers that are commonly used in AD research are those that mimic glucose and index

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metabolic activity ([18F]-2-fluoro-2-deoxy-d-glucose (FDG)), those that bind to Aβ ([11C]-Pittsburg B compound (PIB), [18F]-AV-45 (Florbetapir)), and those that bind to translocator protein and index microglial activation ([11C]-PK11195, [18F]-PBR111, [11C]- and [18F]-PBR28, and [18F]-FEPPA). This is not a complete list, as hundreds of compounds have been developed for the probing of alternative targets in AD, including metal ion chelation in Aβ plaques, CAA, metabotropic glutamate receptors, muscarinic ACh receptors, and several brain enzymes (e.g. GSK-3β, AChE, and monoamine oxidase B (MAO-B) (Holland et al., 2014).

One of the most consistent findings in all of AD biomarker research is that glucose metabolism, as measured by FDG-PET, is reduced in temporo-parietal areas, with impairment greatest in the angular gyrus, and in frontal cortex, though only later in the disease (Herholz, 2003). These regions are myelinated latest in development and are most susceptible to cortical amyloid deposition in AD (Bartzokis, Lu, & Mintz, 2007). FDG-PET discriminatory analyses can identify AD vs. control subjects with 93% accuracy (Herholz et al., 2002). The use of FDG PET is also useful for confirmation of suspected AD subtypes based on hemispheric asymmetries of impaired regions (e.g. left- or right-variant AD), and differentiation between AD and other dementias, as certain regions (basal ganglia, primary motor and visual cortices, and cerebellum) are uniquely spared in AD (Herholz, Carter, & Jones, 2007). FDG-PET may have value as a marker of disease progression and treatment efficacy, as regional signal differences are indicative of conversion from MCI to AD (Cerami et al., 2015), and significant reductions in metabolism over time have been observed in patients, correlating with worsening cognitive performance (G. E. Alexander, Chen, Pietrini, Rapoport, & Reiman, 2002; Jagust, Friedland, Budinger, Koss, & Ober, 1988; R. Mielke, Herholz, Grond, Kessler, & Heiss, 1994).

Since the first human study of the Aβ -binding PIB-PET radiotracer by Klunk et al. in 2004 (Klunk et al., 2004), studies have repeatedly found elevated levels of Aβ throughout the brain (though not in primary sensory and motor cortices) in AD subjects vs. controls (Edison et al., 2007; Clifford R. Jack & Holtzman, 2013; Kemppainen et al., 2006; Nordberg, 2004). In contrast to FDG-PET, PIB-PET may not have the longitudinal sensitivity to detect disease progression or treatment efficacy; Engler et al. (Engler et al., 2006) have shown that PIB retention is relatively stable in AD subjects after two years, suggesting that Aβ deposition as measured by PIB may reach a plateau early in the disease. Consistent with this, recent trials of anti-amyloid compounds (discussed in Section 1.1.5) have selected symptom-free elderly participants based on positivity

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of Aβ-PET scans (e.g. A4 trials). However, approximately 33% of cognitively normal individuals show Aβ accumulation (Clifford R. Jack et al., 2014; Rowe et al., 2010; Villemagne et al., 2013), and Satlin et al. (Satlin et al., 2014) have recently found that only 60% of subjects with MCI thought to be due to AD show Aβ positivity in trial recruitment. As summarized by Sperling et al. (Sperling, Mormino, & Johnson, 2014), even if some evidence suggest that Aβ positivity may be indicative of subsequent cognitive decline (Y. Y. Lim et al., 2014; Mormino et al., 2014; Vos et al., 2013), the binding of Aβ radiotracers *in vivo* should be considered as a necessary but insufficient driver of AD dementia, and interacting factors (such as genetics and co-morbid pathology) should be the subject of future study. The Aβ-binging radiotracer [18F]Florbetapir ([18F]-AV-45) has recently been adopted by large multi-site consortia (Choi et al., 2012; Clark CM, Schneider JA, Bedell BJ, & et al, 2011; Jagust et al., 2010) due to its favourable pharmacokinetics and binding characteristics (K.-J. Lin et al., 2010), though Landau et al. recently found high correlation between different Aβ PET radiotracers across regions (Landau et al., 2014).

PET imaging of inflammation using radiotracers that bind to translocator protein (TSPO) has found that AD subjects generally show increases in signal compared to controls, and that the effect is most prominent in entorhinal, temporo-parietal and posterior cingulate cortices (Cagnin et al., 2001). This is thought to be due to the strong up-regulation of TSPO by active microglia under inflammatory conditions (M.-K. Chen & Guilarte, 2008). Recent evidence suggests that the second-generation TSPO-binding radiotracer [18F]-FEPPA may have high specificity to neurodegenerative disorders, as no age-related change in binding was seen in healthy elderly (Suridjan et al., 2014). As reviewed by Zimmer et al. (Zimmer et al., 2014), the relatively newer field of measuring neuroinflammation *in vivo* using PET has yet to demonstrate consistency between studies of first and second generation radioligands, though converging evidence from second generation studies suggests that increased TSPO expression by active microglia likely occurs after AD onset and may continue to change as the disease progresses (Yasuno et al., 2012). Importantly, the rs6971 polymorphism in the *TSPO* gene has a strong influence on binding affinity of second generation TSPO radiotracers (Owen et al., 2012), and thus must be considered in PET studies of such ligands (this gene variant is the subject of investigation in Chapter 5).

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The latest advance in PET imaging of AD is the development and testing of radiotracers specific for tau protein (recently reviewed by Watanabe et al. (Watanabe, Ono, & Saji, 2015)). Several difficulties have hindered progress on this front including the intracellular localization of NFTs, the co-localization of PHFtau with Aβ plaques, and the lower concentration of tau compared to Aβ in the AD brain (Villemagne et al., 2012). Several promising compounds ([18F]-T807, [18F]-T808, [18F]THK5105, and [18F]-THK523) have advanced from animal to human studies, with excellent *in vivo* properties and high selectivity for PHFtau over Aβ plaques (Chien et al., 2013; Fodero-Tavoletti et al., 2011). Clinical studies will be required to demonstrate the value of tau radiotracers in AD research.

In summary, PET imaging allows for the regional and quantitative measurement of glucose metabolism, amyloid pathology, and neuroinflammation in living human brain. New developments with tau radiotracers may soon offer even greater temporal resolution of the AD process *in vivo*. Unfortunately, the use of PET in routine clinical settings is currently cost prohibitive.

1.3.5 MRI Volumetry and Cortical Thickness

Since AD is a neurodegenerative disorder, it follows that changes in the volume of brain structures vulnerable in the disease may be measurable over time. Magnetic resonance imaging (MRI) is capable of non-invasively mapping the human brain in living subjects, making such measurements possible. While a full discussion of MR physics is beyond the scope of this review, it is important to understand the basics in order to appreciate its complexities and challenges. In MRI, the "spin" of protons (the nuclei of hydrogen atoms which are found abundantly in biological tissue – water, proteins, fats etc.) align to a strong primary magnetic field (termed B0) running along and through the bore of the scanner. When hydrogen atoms within the scanner bore (e.g. those within a subject's brain) are perturbed by a radiofrequency pulse, their spin direction is offset as they absorb the energy. As they "relax" to their original alignment with B0, the protons release their absorbed energy at a rate that is dependent on the T1 and T2 relaxation properties of the tissue in which they reside (these properties are dependent on the biochemical composition of the tissue). The T1 parameter influences the time it takes for the pole of the perturbed proton to return to alignment with B0, whereas T2 affects the component of

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"relaxation" perpendicular to the B0 field (which is caused by the process of precession, where offset spins returning to equilibrium results in a conical trajectory, opposite to a spinning top losing its momentum). The released energy generates voltage in radio antennae coils (a head coil for brain scans) surrounding the subject, which constitute the MR signal that is effectively translated using the Fourier transform to reconstruct images. Depending on the time at which an image is sampled, and other properties of the acquisition sequence, the combination of T1 and T2* signal (T2* indicating total regional T2 signal including influence by phase asynchrony of precessing protons that affect the T2 signal) will be different and result in different contrast between tissues. For full detail on MR physics and MRI, see McRobbie et al. (McRobbie, Moore, Graves, & Prince, 2006)

MRI volumetry is a field concerned with identifying boundaries of distinct brain lobes, regions, and sub-regions in MR images (including pathologies such as white matter hyperintensities, discussed in Section 1.3.6) and extracting estimates of their volumes for association with other variables of interest such as diagnosis, age, or genetic mutations. Typically this is achieved by creating brain atlases (essentially composite 3-D MRI images that are manually labelled with known regions of interest (ROIs)) and aligning new brain images (i.e. those of study subjects) to them. Based on the deformation (stretches, shears and other manipulations that are performed by a process called image registration) required to match the new image to the atlas, it is possible to identify which regions on the new unlabeled image are occupied by regions that have been labelled on the atlas, allowing for ROI volume estimation in the original ("native") space of the new image. This technique can be applied to different image contrasts to identify the volumes of brain structures, tissue types, lesions, or fluids. Using algorithms that manipulate 3-D surface meshes to fit into the boundaries of gray and white matter in the brain, it is possible to estimate the distance between these surfaces at any given point across the cortex, yielding measures of cortical thickness that are also useful for detecting neurodegeneration.

As neuropathology develops in AD, specific cortical and subcortical regions show signs of atrophy; indeed reductions of both volume and thickness of entorhinal cortex, hippocampus, posterior cingulate cortex, and supramarginal gyrus as measured by structural MRI are consistently reduced in AD and MCI vs. controls (Desikan et al., 2009). The pattern of atrophy matches the topology of neuropathology in AD (Dickerson et al., 2009; Dickerson & Wolk, 2012) and is paralleled by loss of neurons (Bobinski et al., 2000; Zarow et al., 2005) and

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increases in NFT deposition (J. L. Whitwell et al., 2008). Reductions in the size of the hippocampus, a hub of memory and learning in the brain (Bliss & Collingridge, 1993), is certainly the most common finding in AD (McConathy & Sheline, 2015), with volume loss occurring presymptomatically and progressing over the course of illness (Fox et al., 1996; Schuff et al., 2009). Discriminant analyses using average cortical thickness over the parahippocampal gyrus was able to identify AD patients vs. controls with 94% accuracy, whereas analyses of a much more focal region (a single vertex in the entorhinal cortex) yielded accuracy of 100% (Lerch et al., 2008). As reviewed by Frisoni et al. (Frisoni, Fox, Jack, Scheltens, & Thompson, 2010), atrophy measured by structural MRI correlates with cognition both cross-sectionally and longitudinally, with changes in focal (hippocampal pathway) and more global brain (total brain and ventricles) volumes correlating closely with cognitive performance over the progression of AD. However, it has also been shown that atrophy does not correlate with postmortem Aβ load (Josephs et al., 2008), suggesting that atrophy is a later consequence of AD indicative of tau-mediated neurodegeneration, and that Aβ measurement (either by PET or in CSF) is a more sensitive marker of progression through pre-AD phases. As such, the neurodegenerative category of AD biomarker defined by Jack and Holtzman (Clifford R. Jack & Holtzman, 2013) encompasses the aforementioned late-occurring CSF T-tau and P-tau, as well as brain atrophy on structural MRI.

Currently, the most widely-accepted temporal model of biomarker change over time (Clifford R. Jack, Knopman, et al., 2013; Clifford R. Jack & Holtzman, 2013) states that Aβ deposition precedes tauopathy, which is followed by neurodegeneration and atrophy (measured by MRI), which then causes cognitive decline. The gap between observable neurodegeneration/brain atrophy and the onset of cognitive decline has been termed “cognitive reserve” (Stern, 2012) (or the ability to maintain normal cognition in the presence of brain pathology or injury), and will be discussed more in Section 7.4.1. While substantial evidence supports this “amyloid-first” model (Clifford R. Jack & Holtzman, 2013), a second model (“neurodegeneration first”) has also been proposed by the same authors (Clifford R. Jack, Wiste, et al., 2013) that captures the literature showing that tauopathy and neurodegeneration may precede amyloid deposition (H. Braak & Braak, 1997) (discussed in Section 1.2.4). Clearly, there is a lack of understanding about the true timing of events leading to and following the onset of AD, and unfortunately structural MRI alone cannot answer these questions.

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Despite consistent observations of brain atrophy in AD, brain structure volumes and cortical thickness is a relatively non-specific marker of neurodegeneration and is seen in the context of normal aging and many other conditions (Dickerson & Wolk, 2012; Fjell et al., 2009; Clifford R. Jack, Knopman, et al., 2013; Murphy et al., 2010). In fact, Fjell et al. (Fjell et al., 2013) found that significant global atrophy (as well as hippocampal atrophy correlating with changes in memory performance) occurred over the course of one year in a group of 132 cognitively normal subjects at very low risk for AD (no signs of MCI within three years).

In summary, volumetric changes in brain structures important for memory and first affected by AD, such as the hippocampus and entorhinal cortex, are reliable indicators of AD risk and progression and thought to represent neurodegeneration. However, the lack of knowledge surrounding other physiological contributors to volume, thickness, and shape of brain structures make mechanistic interpretation of these changes difficult.

1.3.6 White Matter Macrostructure (Hyperintensities, Infarcts)

Using a T2-weighted MRI acquisition sequence that maximizes white matter tissue signal contrast and nullifies the contribution of CSF, it is possible to observe details in the white matter of the brain that would otherwise go undetected. Hajnal et al. (Hajnal et al., 1992) first described an MRI acquisition protocol, called fluid attenuated inversion recovery (FLAIR), with characteristics that produced a low signal in heavily myelinated white matter areas and high signal in unmyelinated regions, and was useful for identifying brain lesions in a range of CNS diseases (Coene et al., 1992; Rydberg et al., 1994), including acute subarachnoid hemorrhage (Noguchi et al., 1995), a type of stroke. Since the beginning of their clinical use, regions of hyper-intense signal (or white matter hyperintensitites (WMH)) in these images have been commonly observed in scans of cognitively normal elderly subjects (prevalence estimates as high as 95% in those aged 65 and over (Longstreth et al., 1996)), but more commonly in MCI and AD subjects than age-matched controls (Bombois et al., 2007; Jellinger & Attems, 2005; Luchsinger et al., 2009). WMH are rare in young healthy individuals (prevalence of ~5%), however, the risk for having WMH increases 10-fold after the age of 55 (Hopkins et al., 2006).

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There are two main commonly observed types of WMH: 1) periventricular, and 2) deep/subcortical (Mäntylä et al., 1997). Periventricular WMH are found outlining the CSF-filled lateral ventricles, while deep/subcortical WMH are more punctate, isolated lesions. As reviewed by Schmidt et al. (R. Schmidt et al., 2011) and Kim et al. (Kim, MacFall, & Payne, 2008), the presence of different types of WMH in different regions correlate with different autopsy-confirmed pathologies and epidemiological risk factors. Smooth periventricular WMH typically have a non-vascular origin; there is little evidence of arteriosclerosis or periarteriolar tissue damage in these regions. It has been suggested that disruption of the ependymal lining of the lateral ventricle, accompanied by gliosis and loss of myelin, may be responsible for these WMH (P. Scheltens et al., 1995). Deep/subcortical WMH represent more ischemic sources of pathology, with the most common being the widening of periarteriolar space, loss of fibres, local atrophy, and arteriosclerosis, and may represent early stage infarcts (early confluent changes) (Kim et al., 2008; R. Schmidt et al., 2011). Molecular examinations of deep WMH show associations with hypoxia-inducible factors (HIFs), suggesting that ischemia resulting from chronic hypoperfusion may be a key contributor to these lesions (Fernando et al., 2006). As such, WMH are often used as indicators of ischemic vascular disease (a type of small vessel disease (SVD)) and show correlation with executive function, attention, and mental flexibility both cross-sectionally and longitudinally (Au et al., 2006; Brickman et al., 2008; Jokinen et al., 2009; Ylikoski et al., 1993). However, only individuals with early confluent or confluent lesions tend to show increases in WMH volume over time, whereas individuals with distinctly punctate lesions do not (R. Schmidt et al., 2003), supporting the notion that confluent deep WMH are those representing underlying vascular irregularities and are thus prone to spreading (R. Schmidt, Petrovic, Ropele, Enzinger, & Fazekas, 2007). Experts have proposed that WMH be sub-classified based on their proximity to the ventricular watershed region (3mm-13mm from ventricular surface) to differentiate between those WMH that are hemodynamically defined (periventricular) and those that are ischemic (deep) (Kim et al., 2008).

A currently unresolved issue in the field is how best to model the effect of WMH; since the presence of WMH is so high in healthy subjects, and the extent of WMH varies so widely between individuals, dose-dependent effects have been demonstrated whereby a certain threshold of WMH volume must be achieved in order for cognitive changes to manifest (Boone et al., 1992). Further, the location of WMH, rather than just total volume, may be an important factor,

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as Brickman et al. have recently shown that parietal WMH specifically are associated with AD (Brickman et al., 2012). Another issue with studying the contribution of WMH to cognitive decline in AD is disentangling its effects from that of brain atrophy; some show that WMH effects on executive function and memory are lost when co-varying from brain volume (DeCarli et al., 1995; DeCarli, Murphy, Teichberg, Campbell, & Sobering, 1996), whereas others show independent effects of both (Swartz, Stuss, Gao, & Black, 2008).

As noted above, punctate deep white matter lesions may represent infarcted tissue. Cerebral infarcts are focal regions of necrotic brain tissue resulting from ischemia (either via the occlusion of blood vessels, embolism, or hemorrhage/stroke) and are the second most common type of pathology observed at autopsy in elderly (Knopman et al., 2003). In 3 397 individuals without a prior history of stroke from the Cardiovascular Health Study (CHS), 28% were found to have infarcts detectable with MRI, and the presence of infarcts was correlated with cognitive and other neurological deficits (T. R. Price et al., 1997). While these "silent infarcts" (i.e. those that do not manifest as clinical emergencies (i.e. stroke)) are quite prevalent in otherwise healthy individuals, their accumulation can cause full-on dementia; in 1974, Hachinski et al. (Hachinski, Lassen, & Marshall, 1974) proposed "multi-infarct dementia" (a type of VaD), which as the name suggests, describes a dementia resulting primarily from the presence of multiple cerebral infarcts.

Infarcts can be classified based on their size (micro or macro), by phase (acute, subacute, or chronic), and by their localization/cause (atherothrombotic, cardioembolic, lacunar, or other) (C. M. Fisher, 1998; NINDS, 1990; E. E. Smith, Schneider, Wardlaw, & Greenberg, 2012). Macro infarcts generally are associated with AD diagnosis and decline in memory (Vermeer et al., 2003), and are associated with the development of MCI from a healthy state and the further progression to moderately impaired states (J. A. Schneider et al., 2009; Yu, Boyle, et al., 2015). As with WMH, the location of infarct may determine the cognitive domain it affects. For example, thalamic infarcts have been shown to preferentially influence memory task performance (Bogousslavsky, Regli, & Uske, 1988; Gold et al., 2005), whereas non-thalamic infarcts affected psychomotor performance (Vermeer et al., 2003). It has also been shown that infarcts are more strongly related to na-MCI, whereas WMH are more strongly related to a-MCI (Luchsinger et al., 2009), and, at the population level, ancestry may influence the prevalence of

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infarcts in AD, whereby those of African-American ancestry are more likely than Caucasians to have mixed pathology (including concomitant infarcts) (Barnes et al., 2015).

The undetected presence of microinfarcts (0.2-2.9mm in size (Arvanitakis, Leurgans, Barnes, Bennett, & Schneider, 2011; Okamoto et al., 2009)) may provide an explanation for why only one or a few macroscopic infarcts can seemingly cause significant cognitive effects; microinfarcts are also thought to arise from small vessel disease (Yip et al., 2005) and have been shown to contribute to risk for cognitive dysfunction independently of other pathologies, including macroinfarcts (E. E. Smith et al., 2012). A common assertion is that the presence of infarcts (indiacative of cerebrovascular pathology) is an additive adjunct to the neuropathological burden of AD (Aβ and PHFtau), resulting in greater risk for clinical AD and subsequent cognitive decline when present in combination, possibly by eroding cognitive reserve (Bangen et al., 2015; Raz, Knoefel, & Bhaskar, 2015; Vermeer, Longstreth Jr, & Koudstaal, 2007).

In summary, white matter hyperintensitites and cerebral infarcts are common and related to AD diagnosis but also show additive effects with AD pathology on cognition. Deep WMH may be a reaction to vascular changes in SVD and useful as a proxy for vascular and inflammatory dysfunction.

1.3.7 White Matter Microstructure (Diffusion Tensor Imaging)

In contrast to coarse macrostuructural measurements of white matter outlined above, the invention of diffusion tensor imaging (DTI) has allowed researchers to examine changes in white matter that are invisible to the naked eye and may precede gross anatomical changes. DTI is an implementation of MRI, using a specific acquisition sequence and magnetic gradients to sample the diffusion characteristics of water in the brain in multiple directions. While diffusion MRI had been introduced in 1986 by Le Bihan et al. (Le Bihan et al., 1986), the application of the mathematical tensor (representing a geometric object, in this case an ellipsoid) to diffusion MRI data to extract the directionality of water diffusion in 3-D was first proposed by Peter Brasser in 1994 (Basser, Mattiello, & LeBihan, 1994). Before this, diffusion could be quantified but only in a single dimension (i.e. orientation of the sample had to be known). Using a specific acquisition sequence called spin-echo, that allows for the measurement of molecular diffusion of liquids

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(developed in 1950 by Erwin Hahn (Hahn, 1950)), and the repetition of this sequence using multiple magnetic field gradients sensitized to diffusion in different directions, it is possible to calculate a diffusion tensor in each voxel of the brain (Basser, 1995). The diffusion tensor is a 3x3 co-variance matrix describing diffusion displacements in 3-D, with eigenvectors ($\hat{e}_1$, $\hat{e}_2$, $\hat{e}_3$) and eigenvalues (λ_1, λ_2, λ_3) describing the directions and apparent diffusivities of water in each dimension (A. L. Alexander, Lee, Lazar, & Field, 2007). If the eigenvalues are approximately equal ($\lambda_1 \sim \lambda_2 \sim \lambda_3$), then diffusion is said to be isotropic (i.e. equal in all directions, as water in a glass, or in CSF), whereas if eigenvalues are quite different in magnitude (e.g. $\lambda_1 > \lambda_2 > \lambda_3$) then diffusion is anisotropic (i.e. directionally unequal, as water flowing through a pipe, or within axons). The principle axis of diffusion corresponds to the eigenvector with the largest corresponding eigenvalue.

In clinical research, information from the diffusion tensor is commonly processed in one of two ways: separately within each voxel to analyze focal white matter microstructural changes, or for the reconstruction of entire white matter fibres using tractography (helpful review by (Snook, Plewes, & Beaulieu, 2007), though somewhat outdated). Voxel-based approaches (such as tract-based spatial statistics (TBSS)) are useful for detecting focal changes in WM microstructure that may only affect one part of an entire WM tract and lend themselves best to hypothesis-free testing of the whole brain, since there is no pre-definition of what constitutes of tract or ROI (S. M. Smith et al., 2006). TBSS can be adapted to produce average diffusion metrics across ROIs using a WM atlas; such a protocol is being used by the Enhancing Neuro Imaging Genetics through Meta-Analysis (ENIGMA) consortium (Thompson et al., 2014) to standardize and simplify image processing across many datasets and facilitate inter-site comparability. Tractography may give a more anatomically accurate assessment of WM integrity since it uses the directional information contained in the tensor to estimate the trajectory of white matter fibres, which then can be used to calculate metrics of diffusion for entire tracts (Voineskos et al., 2009).

The most commonly used diffusion metric in clinical research of AD is fractional anisotropy (FA) (Koay, Chang, Carew, Pierpaoli, & Basser, 2006), which is described by the following equation:

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$$FA = \sqrt{\frac{(\lambda_1 - \mathrm{MD})^2 + (\lambda_2 - \mathrm{MD})^2 + (\lambda_3 - \mathrm{MD})^2}{2(\lambda_1{}^2 + \lambda_2{}^2 + \lambda_3{}^2)}}$$

Where MD (mean diffusivity, also called apparent diffusion coefficient (ADC)) is equal to trace (the sum of the tensor eigenvalues λ_1, λ_2, and λ_3) divided by three. FA is a measure of the directional restriction of water in the voxel, and is commonly used as an indicator of white matter "integrity", sensitive to changes in myelination, extracellular water (as in inflammation or tissue damage), axonal density and diameter (M. Takahashi et al., 2002), and especially fibre orientation within a voxel (Pierpaoli, Jezzard, Basser, Barnett, & Di Chiro, 1996). In contrast, MD is a more general measure of translational diffusion and increases in areas of tissue damage, including phases of ischemia (O'Sullivan et al., 2001). However, due to the wide range of potential contributors to tensor characteristics, the term "integrity" is discouraged by many in the field; the true meaning of changes in diffusion metrics is not always clear (D. K. Jones, Knösche, & Turner, 2013).

Beyond the cumulative influence of vascular changes and ischemia on WM structure in AD, which may be most accurately indexed by deep WMH and infracts (Section 1.3.6), DTI may be sensitive to earlier events in AD pathogenesis, such as the hyperphosphorylation of tau. The tau proteins are primarily found in axons, maintaining microtubule-facilitated axonal transport (Buée, Bussière, Buée-Scherrer, Delacourte, & Hof, 2000), and DTI is particularly sensitive to axonal degeneration. In this way, microstructural WM changes measured with DTI may be a harbinger of localized atrophy that contributes to the onset of typical AD symptoms, especially in tracts connecting to the medial temporal lobe (Bozzali et al., 2012). The literature on FA and MD differences between healthy controls, MCI, and AD subjects shows that changes in these metrics between diagnostic groups is somewhat region-specific (though MD shows more widespread changes than FA), with significant differences in FA commonly observed between AD + MCI vs. controls, but differences in MD between AD vs. controls as well as MCI vs. controls (meta-analysis by Sexton et al. (Sexton, Kalu, Filippini, Mackay, & Ebmeier, 2011) and reviews by Oishi et al. (Oishi, Mielke, Albert, Lyketsos, & Mori, 2011) and Amlien et al. (Amlien & Fjell, 2014)). The regions in which changes are most consistently observed map loosely to those affected early by AD pathology and neurodegeneration: they are tracts extending

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to the entorhinal cortex, hippocampus, and parrahippocamal gyrus (Choo et al., 2010; Fellgiebel et al., 2004; Rose et al., 2006; Salat et al., 2010; Zhou et al., 2008), the temporo-parietal cortex (Bozzali et al., 2002; Medina et al., 2006; S. Takahashi et al., 2002; S. Xie et al., 2006), and posterior cingulate cortex (Choo et al., 2010; Nakata et al., 2008; Y. Zhang et al., 2007; Zhou et al., 2008). The major white matter tracts connecting these regions are the uncinate fasciculus (UF), the cingulum bundle (CB), the inferior longitudinal fasciculus (ILF), and the inferior fronto-occipital fasciculus (IFOF); each have been shown to have reduced "integrity" (i.e. lower FA, higher MD) cross-sectionally (Cho et al., 2008; Taoka et al., 2006). In longitudinal analyses, the UF has shown increases in MD and decreases in FA with worsening AD symptoms (Acosta-Cabronero, Alley, Williams, Pengas, & Nestor, 2012; Kitamura et al., 2013) (UF FA correlates with MMSE and ADAS-cog score cross-sectionally (Morikawa et al., 2010)). Other white matter tracts, such as the superior longitudinal fasciculus (SLF) and the interhemispheric corpus callosum (CC), have also been implicated in AD pathogenesis according to what is known as the "retrogenesis model of AD" (Bartzokis et al., 2007; Reisberg et al., 1999). This hypothesis states that the large-diameter, early-myelinating white matter fibres that are first to develop (such as those comprising primary motor tracts) are also those last affected by age-related AD pathology (as opposed to thinner, late-myelinating tracts, including neocortical association fibres, such as SLF, which are affects first) (Stricker et al., 2009).

While FA and MD are the most commonly reported DTI metrics in clinical research, two other indices derived from the diffusion tensor may represent different underlying microstructural changes: radial diffusivity (RD) (diffusion perpendicular to primary fibre direction; may be most sensitive to demyelination and dysmyelination (S.-K. Song et al., 2002)) and axial diffusivity (AxD) (diffusion parallel to primary fibre direction; may be most sensitive to axonal damage (DeBoy et al., 2007; S.-K. Song et al., 2003).

The choice of diffusion metric analyzed may have important implications for study results; a preliminary analysis of 155 subjects from the Alzheimer's Disease Neuroimaging Initiative (ADNI, phase 2) showed that MD, RD, and AxD were more sensitive than FA in differentiating between AD, MCI, and controls, using both voxel-wise and ROI-based approaches with TBSS (Nir et al., 2013). Other newer metrics describing the diffusion properties of brain tissue are microFA (which can differentiate between microstructural shape contributions to diffusion signal (e.g. different cell morphologies) (Lasič, Szczepankiewicz, Eriksson, Nilsson, & Topgaard,

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2014)), dispersion and curvature (descriptions of fibre geometry (Savadjiev, Kindlmann, Bouix, Shenton, & Westin, 2010), and free water (a correction for FA that removes isotropic signal due to fluid partial volumes (Pasternak, Sochen, Gur, Intrator, & Assaf, 2009)).

A major limitation of existing DTI approaches is that the complex architecture of white matter fibres in the brain often cannot be captured by a single diffusion tensor in one voxel that may span several millimetres in three dimensions (fibres often cross within single voxels). As a result, FA may be very low in a region of highly intact, cohesive white matter fibres that are crossing. To address this issue and others, more recent diffusion imaging methods are being explored; they include q-ball imaging (QBI) (Tuch, Reese, Wiegell, & Wedeen, 2003), high angular diffusion imaging (HARDI) (Frank, 2002), and diffusion spectrum imaging (DSI) (Wedeen, Hagmann, Tseng, Reese, & Weisskoff, 2005).

In summary, microstructural qualities of white matter can be assessed using diffusion tensor imaging and may be indicators of neurodegeneration found in AD. While changes in common diffusion metrics are consistently seen in AD-affected brain regions, the interpretation of underlying mechanisms remains mostly speculative. New diffusion MRI-based techniques may improve our understanding of microstructural white matter changes in the near future.

1.3.8 Major Biomarker Studies

A number of landmark studies have operated over several decades with the aims of identifying the underlying causes and sources of heterogeneity in AD by administering a wide array of biomarker tests to well-characterized, large groups of people and performing longitudinal follow-up. They are of two main types: prospective cohort studies in the general population and longitudinal studies of defined target populations. While these are not the only such studies, three major efforts that are analyzed in this thesis are the Alzheimer's Disease Neuroimaging Initiative (ADNI), the Religious Orders Study (ROS), and the Memory and Aging Project (MAP).

The Alzheimer's Disease Neuroimaging Initiative (ADNI) is an international collaboration of 59 universities, health centres, and hospitals in the United States and Canada with the primary goal of detecting AD at the earliest possible stages and identifying informative biomarkers for disease progression (Mueller et al., 2005). To date there have been over 600 publications using

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ADNI data (the impact of ADNI was recently reviewed by Weiner et al. (Weiner et al., 2015)). The original study (ADNI 1) began in October 2004, under the leadership of principal investigator Michael Weiner (University of California, San Francisco), with $67 million in funding from the NIA, the National Institute of Biomedical Imaging and Bioengineering (NIBIB), 13 private companies, and two foundations, and was planned to run for five years (Mueller et al., 2005). This was a time when neuroimaging and CSF biomarker research in AD was just beginning to show promise, and ADNI sought to become the largest longitudinal cohort study in history to collect multi-modal neuroimaging (structural MRI and PET), genetic, cognitive, clinical, CSF, and blood-based biomarker data in the same set of subjects, with follow-up (the original goal was to recruit 800 subjects in total; 200 cognitively normal elderly, 400 AD, and 200 MCI). Furthermore, one of its primary aims was to make all data and methods publically available to researchers worldwide. The original study design included baseline screening and 6- and 12-month follow-up for clinical and biomarker assessments. However, as the study grew during a period of rapid progress in AD research, it became apparent that more study subjects and additional biomarkers were required to address developing hypotheses more conclusively (Weiner et al., 2010, 2012). Therefore, in 2009, ADNI was extended into the ADNI GO phase (by a two-year NIH Grand Opportunities grant; hence GO), to recruit 200 new subjects with early MCI and continue follow-up on 500 controls and MCI subjects from ADNI 1. Finally in 2010, ADNI funding was renewed (with another $67 million) and the ADNI 2 study phase began, with the goal of recruiting 650 new subjects at varying stages of AD (as well as continue follow-up on portions of ADNI 1 and ADNI GO subjects). The ADNI 2 protocol introduced DTI and [18F]Florbetapir PET for all subjects. ADNI as it is referred to in this thesis is in fact the North American ADNI, the founding member of the World Wide ADNI (WW-ADNI) umbrella organization that includes ADNIs from seven other countries and continents (Europe, Japan, Australia (AIBL), Taiwan, Korea, China, and Argentina (Brazil will soon join as well)).

The Religious Orders Study (ROS) is an ongoing NIA-funded study headed by David Bennett and centered at the Rush University Medical Center (Chicago, IL, USA). Motivation for the study came from a lack of prospective, longitudinal, autopsy follow-up studies of elderly with normal cognition prior to 1993. Very few had been published (Berg, McKeel, Miller, Baty, & Morris, 1993; Crystal et al., 1993; Katzman et al., 1988), all with very small sample sizes ($n<20$), and the trend toward single case studies on AD pathologies in non-demented elderly did not aid

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the generalizability of findings in this area. To begin answering questions surrounding the differences (and similarities) between healthy aging and AD, David Bennett and colleagues at Rush University designed a study similar to David Snowdon's Nun Study. The Nun Study, based out of the University of Kentucky, was funded by the NIA and private donors in 1986 as a pilot study and was expanded in 1990 to enroll older members of the School Sisters of Notre Dame (Mankato, MN, USA) (Snowdon, 1997). It followed 678 sisters (aged 75 and over), most cognitively normal at enrollment, all of whom had agreed to postmortem autopsy for the sake of education and scientific discovery. Accordingly, the ROS was proposed in 1992 to follow members of over 40 religious communities across the US and was funded as a Core of the Rush Alzheimer's Disease Core Center in 1993, with enrollment beginning in 1994 (Bennett, Schneider, Arvanitakis, & Wilson, 2012; Wilson, Bienias, Evans, & Bennett, 2004). Whereas the Nun Study by design enrolled only women, the ROS initially sought to enroll over 1000 women and men. The goals of the study are threefold: 1) identify biomarker associations with AD, MCI, and cognitive decline proximate and years prior to death, 2) identify risk factors for AD, MCI, and cognitive decline incidence, and 3) model the neurobiological links between disease risk and clinical symptoms. A fringe benefit (or, depending on the analysis, a potential confound) of analyzing a sample from a uniquely healthy population is the opportunity for gaining insight into resilience mechanisms of aging, an active area of research with ROS data (Negash et al., 2013; Negash, Bennett, Wilson, Schneider, & Arnold, 2011). Currently, over 1 100 participants have been enrolled in ROS, and the study is planned to conclude in June 2016.

The Memory and Aging Project (MAP) is a study of the same design as ROS (healthy elderly subjects evaluated longitudinally with brain donation and autopsy at death), except with subjects drawn from a different, more general community-based target population (Bennett et al., 2005). In fact, the only exclusion criteria is the inability to sign the Anatomical Gift Act, reducing the "healthy volunteer effect" (Lindsted, Fraser, Steinkohl, & Beeson, 1996). Recruitment for MAP began in 1997 at over 30 residential facilities across northeastern Illinois and is ongoing. Compared to other cohort studies, the recruitment of subjects primarily from continuous care retirement communities has resulted in higher study follow-up and autopsy rates. The amount and variety of phenotypes that are assessed as part of the ROS and MAP studies are impressive. The list includes but is not limited to: clinical diagnoses, cognitive performance, motor function and physical frailty, sleep duration and quality, gait, pulmonary function, reports of daily living

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(including physical activity), ante-mortem biospecimen collection (blood for biomarkers and genomics), ante-mortem neuroimaging (MRI, DTI, and PET), and postmortem biospecimen collection (for future analyses) and immediate neuropathological evaluation. The combined ROS and MAP dataset is immense in size, but more importantly uniquely rich and deeply phenotyped, providing statistical power for a wide range of study types and integrative analyses.

In summary, biomarkers are important for the identification of risk for AD, the diagnosis of AD, and potentially the tracking of efficacy of treatments in clinical trials. Specific combinations of biomarkers observed in AD and in healthy individuals provide hints as to underlying pathogenesis and offer insights into the heterogeneity of the disorder, even beyond more clear-cut subtypes (outlined in Section 1.1.3). In this thesis, robust biomarkers of multiple AD-related mechanisms are measured and analyzed in healthy and AD subjects across the human lifespan to draw conclusions about the functional significance of genetic variants that that influence these mechanisms through shared pathways.

1.4 Alzheimer's Disease Genetics

1.4.1 Background

Notwithstanding the cumulative environmental and lifestyle factors that influence risk for AD (Discussed in Section 1.1.5), biological risk pathways for AD begin at the genetic level, with consequent impact on mRNA, protein, brain structure, brain circuitry and then cognitive and behavioral impairment. While the early onset FAD is an autosomal dominant condition known to be caused by mutations in *APP*, *PSEN1*, and *PSEN2* genes, late onset AD has a considerably more complex genetic foundation (reflected by the range of potential etiopathologies and contributors to heterogeneity). The first reports suggesting a genetic component to AD came in the early 1980s (R. Harris, 1982); Albert Heyman's analyses of 68 AD patients and their families provided pivotal evidence for the familial aggregation of AD, where the prevalence of AD in the families of patients was much higher than in the general population (Heyman et al., 1983). The most accurate estimates of AD heritability, which is the proportion of phenotypic variance (i.e. having a diagnosis of AD or not) that can be attributed to genetics rather than environment, are often cited as those from the Swedish Twin Registry of 392 twin pairs with AD (Gatz et al.,

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2006), and suggest that approximately 80% of the risk for developing AD may be due specifically to genetic factors.

Before the successful sequencing of the human genome, genetic linkage analysis was used to identify genomic regions implicated in heritable diseases (Dawn Teare & Barrett, 2005). Studies using this method take advantage of a phenomenon called genetic linkage, whereby the physical distance between points on a chromosome (e.g. genes or genetic markers (variants)) determines the likelihood that they will be separated by crossover events between chromosomes during meiosis (recombination). When the rate of recombination between genetic "markers" is less than 50%, it can be inferred that they are on the same chromosome (discovered by Thomas Morgan and Alfred Sturtevant (Morgan, 1911; Sturtevant, 1913)). One common type of early genetic marker used in linkage studies cleverly harnesses the intrinsic functions of simple DNA-cutting enzymes known as restriction endonucleases; the presence or absence of certain nucleotides within their target sites would determine whether the DNA molecule was cleaved at that location (known as restriction fragment length polymorphism (RFLP)), producing DNA fragments of differing sizes and providing an indirect way of detecting sequence variation. Positions in the genome where one nucleotide may be substituted for another are referred to as single nucleotide polymorphisms (SNPs), and different sequence variants of the same genomic region (locus) are referred to as alleles; for example, individual A with a [C] nucleotide at SNP position #1 has a different allele than individual B, who has a [T] nucleotide at the same SNP position #1. By comparing the proportion of offspring in families that possess recombinant vs. parental alleles (i.e. estimating the recombination rate), a "map" distance between two markers (in centimorgan (cM) units) can be calculated. By combining this genotypic linkage information, which allows for the tracking of alleles between generations, with phenotypic information, such as AD diagnosis, the approximate location of causal genetic variants that co-segregate with a genetic marker can be determined (Pulst SM, 1999).

As a major landmark in human biological research, the first drafts of the human genome sequence were published in 2001 by independent efforts led by Eric Lander, then of the Whitehead Institute of Biomedical Research and a key member of the publicly funded Human Genome Project (Lander et al., 2001), and Craig Venter, of the private biotechnology company Celera (Venter et al., 2001), in Nature and Science on February 15th and 16th, respectively. The final non-draft version of the sequence was completed by the Human Genome Project in 2003

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(13 years after the project began) and published in 2004 (International Human Genome Sequencing Consortium, 2004), with an error rate of 1/100 000 nucleotide bases, estimating that the 2.85 billion nucleotide genome contained 20 000-25 000 protein-coding genes. This accomplishment effectively popularized the genetic association study, which uses more finely-mapped physical chromosomal coordinates, rather than estimates of recombination rates in families, to link phenotypes directly to allele frequencies in samples of unrelated individuals. Polymerase Chain Reaction (PCR)-based genotyping methods using sequence-specific DNA primers for the tagging of single nucleotide polymorphisms became a popular tool for performing such studies, facilitating primarily hypothesis-driven investigations of candidate genes within reagions that had been implicated by linkage studies (C. M. Lewis & Knight, 2012). The Human HapMap project (Gibbs, Belmont, Boudreau, Leal, & al, 2005), which sought to generate a database of known positions of common variation in the human genome, was launched in 2002 and facilitated the development for chip-based microarray genotyping methods, which allows for the testing of millions of SNP-phenotype associations simultaneously. The subsequent lowering of costs for genome-wide genotyping led to the era of GWAS (Hirschhorn, 2009; Keller et al., 2007).

1.4.2 Genome-wide Association Studies (GWAS) in AD

The first GWAS in AD was published by Grupe et al. in 2007 (Grupe et al., 2007), who tested 17 343 SNPs using a multi-tier approach in 1 808 AD cases and 2 062 controls. An additional 10 case-control and family-based AD GWAS were published between 2007-2009 (see Ertekin-Taner for comprehensive review (Ertekin-Taner, 2010)), culminating in back-to-back large scale GWAS (Harold et al., 2009; Lambert et al., 2009), each of well over 10 000 subjects, both uncovering *CLU* (encoding clusterin, or ApoJ) as a risk locus. Since these first large-scale GWAS, seven increasingly large original and meta-analytic studies have been published (Hollingworth et al., 2011; X. Hu et al., 2011; Lambert et al., 2013; Miyashita et al., 2013; Naj et al., 2011; Wijsman et al., 2011), culminating in a recent 74 000 subject GWAS meta-analysis by the IGAP (Lambert et al., 2013). Altogether, there are approximately 25 independent genome-wide significant loci for AD diagnosis. GWAS of secondary AD phenotypes (such as age-at-onset (Kamboh et al., 2012; Thambisetty, An, & Tanaka, 2013)) and biomarkers (including

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hippocampal volume (Bis et al., 2012; Furney et al., 2011; Melville et al., 2012; Stein et al., 2012), white matter microstructure (Jahanshad, Rajagopalan, et al., 2013), white matter hyperintensities (Fornage et al., 2011), cognition (G. Davies et al., 2014; Sherva et al., 2014), and AD neuropathology (Beecham et al., 2014; Cruchaga et al., 2013; Ramanan et al., 2014; Shulman et al., 2013)) have yielded mixed results, with very little overlap between findings (other than *APOE*).

While GWAS can serve as a platform for discovery of genomic regions related to AD, they are limited by intrinsic difficulties with statistical power and the debatable validity of their original underlying assumptions. When originally conceived, the hope was that GWAS could identify all sources of heritability for AD and other complex diseases; however, common SNPs genome-wide (2 046 116 imputed and genotyped) explain only 33.1% of variance in AD (compared to heritability estimates of ~80% (Gatz et al., 2006)) (Ridge, Mukherjee, Crane, Kauwe, & Alzheimer's Disease Genetics Consortium, 2013). This discrepancy is common among complex illnesses and known as the problem of "missing heritability" (Manolio et al., 2009). There have been multiple proposed explanations for this missing heritability (reviewed by expert panel in (Eichler et al., 2010)), these commonly include: 1) the underestimation of effect sizes due to incomplete linkage disequilibrium (LD) between GWAS SNPs and causal variants , 2) undetected contributions of rare variants (i.e. "common disease, common variant" hypothesis vs "common disease, rare variant" hypothesis (N. J. Schork, Murray, Frazer, & Topol, 2009)), 3) overestimates of heritability due to genetic interactions, epigenetics, or gene-environment interactions (Zuk, Hechter, Sunyaev, & Lander, 2012), and 4) the presence of many remaining undetected variants of small effect. While advances in statistical genomics have improved the discovery capacity of GWAS with methods that allow for the incorporation of *a priori* information (Gagliano, Barnes, Weale, & Knight, 2014; A. J. Schork et al., 2013) to alleviate multiple testing burdens (Sun, Craiu, Paterson, & Bull, 2006; C. Xu, Ciampi, & Greenwood, 2014), the multi-genic, highly heterogeneous nature of clinical AD is not well-suited to the SNP-diagnosis association paradigm; sample sizes required to detect all contributing variants (many with questionable effect sizes) are unfeasibly large.

In Summary, GWAS have played a key role in both the discovery of new AD risk genes and validation of existing associations, though they suffer from many fundamental methodological

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and conceptual limitations. While genome-wide genotype data will no doubt play important roles in future study designs, it is likely that the archetypal diagnostic GWAS has reached its potential.

1.4.3 After GWAS: Focusing on Promising Candidates

Addressing the issues of GWAS and making progress in the "post-GWAS" era (Q. Huang, 2015; Visscher, Brown, McCarthy, & Yang, 2012) requires more directed approaches to understand the functional consequences of genetic variation implicated in disease (Stranger, Stahl, & Raj, 2011). The combining of genetics with informative quantitative phenotype data rather than diagnostic status typically demonstrates a several fold larger effect of the risk gene on a specific brain structure or other biomarkers, as compared to a clinical phenotype (Meyer-Lindenberg, 2010; Meyer-Lindenberg & Weinberger, 2006; Voineskos et al., 2011). When applied in presymptomatic individuals, the imaging-genetics approach has the potential to inform where, when, and how a gene may exert risk for AD. The genes examined in this thesis (*APOE*, *SORL1*, *TSPO*, and *BDNF*) are not an exhaustive list of AD risk loci by any means. Rather, they were chosen carefully based on a priori knowledge of their functional roles in each of the AD hypotheses/mechanisms described above (Section 1.2). Only two of these genes have been identified by GWAS for AD (*APOE* and *SORL1*), however, as outlined above, it is no surprise that genes important in AD etiopathogenesis may not be identified by GWAS since the complex nature of their contribution cannot be captured by such a simplistic disease model. Candidate gene approaches are essential in the field not only for necessary validation, replication, and in-depth exploration of GWAS associations, but also for discovery; *SORL1* is just one example a gene that was implicated in AD (Rogaeva et al., 2007) prior to its discovery by GWAS (Feulner et al., 2010).

1.4.4 Apolipoprotein E *(APOE)*

APOE is the most well-established and impactful genetic risk factor for late-onset AD, and was discovered by Allen Roses' group at the Duke University Alzheimer's Disease Research Center (ADRC) in the early 1990s (Corder et al., 1993; Saunders et al., 1993; Strittmatter et al., 1993).

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The association of *APOE* with AD has since been replicated near-universally and across racial backgrounds, with one copy of the ε4 allele conferring ~3x risk, and two copies ~13x risk for AD in Caucasians (Farrer et al., 1997; Sadigh-Eteghad, Talebi, & Farhoudi, 2012). The number of *APOE* ε4 alleles also correlates with earlier age-at-onset of AD (Corder et al., 1993; Rebeck, Reiter, Strickland, & Hyman, 1993). While early results for the association of *APOE* with AD in African American (AA) populations were mixed (D. A. Evans et al., 2003; M. X. Tang et al., 1998), recent larger studies, including the largest AA population GWAS to date (n=5 896) (Reitz et al., 2013) and longitudinal analyses in AA and Yoruba (from Southwestern and North central Nigeria) (Hendrie et al., 2014), have confirmed its risk-conferring effect across all ethnic groups, albeit with different odds ratios (ORs) (homozygous ε4 vs. non-ε4 $OR_{Hispanic}$~2.2, OR_{AA}~5.7, $OR_{Caucasian}$~13, $OR_{Japanese}$~33) (C.-C. Liu, Liu, Kanekiyo, Xu, & Bu, 2013). In individuals with AD, the estimated prevalence of *APOE* ε4 carriers is ~49-62% (meta-analysis of 139 studies (A. Ward et al., 2012)), whereas in the general population, the prevalence is much lower, at ~6-20% (meta-analysis of 199 studies (P. P. Singh, Singh, & Mastana, 2006)) (Rebeck et al., 1993).

An advantage of biomarker studies of genetic risk factors is that at-risk groups can be identified at any age, regardless of other factors that are influenced by age or environment (e.g. sub-clinical accumulation of pathology). Studies of *APOE* ε4 status in relation to brain structure are very inconsistent (reviewed by (Fouquet, Besson, Gonneaud, La Joie, & Chételat, 2014)), with groups reporting lower (K. Chen et al., 2007; Lind et al., 2006; Plassman et al., 1997), higher (Honea, Vidoni, Harsha, & Burns, 2009; Striepens et al., 2011), and no differences (Filippini et al., 2011; Hostage, Roy Choudhury, Doraiswamy, Petrella, & for the Alzheimer's Disease Neuroimaging Initiative, 2013; C. R. Jack et al., 1998; Reiman et al., 1998; H. Schmidt et al., 1996) in gray matter volumes between ε4 carriers vs. non-carriers. One explanation for inconsistent findings of *APOE* in imaging-genetics is that ε4 is a mediator, rather than a driver of AD risk, which requires additional pathological insults (M. M. Mielke et al., 2011) or genetic risk factors (Yajima et al., 2015) to manifest. Another potential explanation is that its effects on cognition (Seeman et al., 2005), and both functional (Nichols et al., 2012) and structural (Lind et al., 2006; Schuff et al., 2009) brain imaging may be age-dependent (Nichols et al., 2012). This will be explored further in Chapter 4 of this thesis.

The mechanism by which *APOE* confers these aforementioned changes, and ultimately risk for disease, is not well understood. The APOE protein is composed of 299 amino acids and has a

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molecular mass of ~34kDa, with its genetically-determined isoforms corresponding to differences at positions 112 and 158: APOE2 (Cys112, Cys158), APOE3 (Cys112, Arg158), and APOE4 (Arg112, Arg158). In the CNS, APOE is normally expressed in glial cells and astrocytes, can be expressed in neurons under stress conditions (Q. Xu et al., 2006), and has important functions in cholesterol transport, nerve regeneration, the immune response, and scavenging toxins (Robert W. Mahley, 1988; Robert W. Mahley, Weisgraber, & Huang, 2006; R. W. Mahley & Rall, 2000). Transgenic mice lacking *APOE* show accumulation of cholesterol-rich very low density lipoproteins (VLDL) and develop severe atherosclerotic lesions (Nakashima, Plump, Raines, Breslow, & Ross, 1994; Reddick, Zhang, & Maeda, 1994). The APOE4 isoform possesses two characteristics that have been proposed to underlie its deleterious effects in relation to the "neutral" APOE3: 1) lack of stability and 2) domain interaction between amino- and carboxyl-terminals that influences binding properties (Nguyen et al., 2014). Its lack of stability means that APOE4 is more likely found in a molten globule (unfolded, reactive intermediate) form (Morrow et al., 2002) and is more readily degraded (H. Xu et al., n.d.) than other APOE isoforms, resulting in increases in lipid binding and membrane disruption, as well as other potentially pathogenic functions (Robert W. Mahley & Huang, 2006). In terms of domain interaction and resulting differences in structural conformation, it has been shown that mutant APOE4 lacking domain interaction properties behaves like APOE3 (Dong et al., 1994), and that interruption of domain interaction in APOE4 by small molecule disruption (a structural corrector known as CB9032258) restores mitochondrial and neurite outgrowth deficits in neurons *in vitro* (H.-K. Chen et al., 2012). APOE4 preferentially binds VLDL, whereas APOE3 binds high density lipoproteins (HDL), and thus APOE4 has been associated with hyperlipidaemia, hypercholesterolemia, atherosclerosis, heart disease, and stroke (Kalmijn, Feskens, Launer, & Kromhout, 1996; Lahoz et al., 2001); ε4 is primarily considered a vascular risk factor. This vascular risk conferred by *APOE* ε4 may act synergistically with other risk factors such as type 2 diabetes and peripheral vascular disease to increase risk for AD (Haan, Shemanski, Jagust, Manolio, & Kuller, 1999; Peila, Rodriguez, Launer, & Honolulu-Asia Aging Study, 2002). Further support that APOE exerts AD risk via primarily vascular mechanisms comes from functional MRI studies where disruption of the resting state connectivity was observed in ε4 carriers in the absence of AD pathology (Sheline et al., 2010).

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APOE may also influence amyloidogenic mechanisms; ε4 has been consistently associated with increased Aβ deposition in healthy, MCI, and AD subjects measured by CSF (Morris et al., 2010) and PIB-PET imaging (Reiman et al., 2009; Villemagne et al., 2011), and may increase β-secretase activity (Ewers et al., 2008). APOE was originally thought to bind directly to Aβ to facilitate its clearance from the brain. In fact, the serendipitous observation that APOE protein immunoreactivity was associated with amyloid plaques and CAA in AD brains led to the discovery of the ε4 risk factor in the first place (Namba, Tomonaga, Kawasaki, Otomo, & Ikeda, 1991). However, it was shown by Verghese et al. (Verghese et al., 2013) that APOE does not bind well to Aβ in solution, but rather it competes with Aβ for low-density lipoprotein receptor-related protein (LRP1)-mediated uptake by astrocytes, thus showing indirect regulatory effects on Aβ clearance. *APOE* ε4 has also been associated with P-tau, though not as consistently (Brecht et al., 2004; Morris et al., 2010). Another mechanism via which APOE may influence risk for AD is via alterations in brain activity and structure; neuronal activity is shown to regulate the levels of interstitial fluid Aβ in AD-vulnerable brain regions (Bero et al., 2011). It has also been shown that the effects of *APOE* ε4 on core AD pathology in mice may be greater in females than males (Alexandra Moser et al., 2015), adding to the complexity surrounding *APOE*'s effects on AD phenotypes.

In summary, the *APOE* ε4 allele is undisputedly the strongest known genetic risk factor for late onset AD. However, the mechanism by which it confers risk is poorly understood. Evidence shows that *APOE* ε4 status may influence brain structure and cognition as early as childhood, however the temporal effects of this gene are not known as studies continue to find conflicting results.

1.4.5 Sortilin-Like Receptor *(SORL1)*

SORL1 (also known as LR11 and SORLA) is another highly-studied AD risk gene important for intra-celluar trafficking of APP and lysosomal targeting of Aβ (for comprehensive review, see Thakurta et al. (Thakurta & Andersen, 2015)). The SORL1 protein was first identified as a candidate for involvement in AD by Scherzer et al. (Scherzer et al., 2004), who observed that levels of the protein were twofold lower in lymphoblasts of AD patients vs. controls, as well as reduced by ~25% in neurons (but not glia). Subsequent studies confirmed this decrease in both

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AD (Ma et al., 2009) and MCI (Sager et al., 2007) patients. The genetic association of *SORL1* with AD was discovered by Peter St. George Hyslop's group, based at the University of Toronto and in collaboration with Boston University (Boston, MA) and Columbia University (New York, NY), the same responsible for the discovery of *PSEN1* (Sherrington et al., 1995) and *PSEN2* (Rogaev et al., 1995) mutations in FAD. Hyslop was interested in testing associations between AD and candidate genes coding for proteins involved in vesicular sorting of APP; specifically, the vacuolar protein sorting (VPS) family of proteins, SORL1 included, which had recently been implicated in AD by gene-expression profiling (S. A. Small et al., 2005). In multiple datasets, including two from Northern European and Caribbean families, two Israeli Arab and North European case-control samples, and a fifth independent Caucasian replication sample from the Mayo Clinic, they found that SNPs in the same two regions of *SORL1* (5' haplotypes primarily in non-Europeans and 3' haplotypes in Europeans) were consistently associated with AD (Rogaeva et al., 2007). They went further to perform exon sequencing of *SORL1*, finding no rare pathogenic variants, which suggests that the causal variants were either genotyped directly or contained within introns. Finally, they performed cell biology experiments to demonstrate that individuals with AD risk haplotypes showed reduced expression of *SORL1* mRNA in brain tissue and that SORL1 protein binds to APP, directing its processing to endocytic (amyloidogenic) or recycling (non-amyloidogenic) pathways. The arbitrary numbering convention applied to SNPs in this seminal study (SNPs 1-29) has been preserved by the field and is still commonly used in publications today. Shortly after, *SORL1* was identified as significant by a meta-analytic GWAS (Feulner et al., 2010), and positive replications in larger samples and different ethnic groups began to accumulate (Kölsch et al., 2009; J. H. Lee et al., 2007; F. Liu et al., 2009, 2009; Ning et al., 2010; Tan et al., 2009). *SORL1* has since become one of the most widely acknowledged AD risk genes in the field (meta-analysis (Reitz et al., 2011)).

SORL1 helps direct the preferential transport of amyloid precursor protein (APP) to endosomal recycling pathways, away from beta-secretase cleavage and subsequent $A\beta_{40}$ and $A\beta_{42}$ formation (Andersen et al., 2005; Offe et al., 2006). *In vitro* studies have found that increased levels of SORL1 result in decreased APP processing (V. Schmidt et al., 2012) and greater production of intracellular $A\beta_{42}$ (Ma et al., 2009; V. Schmidt et al., 2012). Disruption of SORL1 can also influence tau-related cellular processes (Capsoni et al., 2013). *In vivo*, data from AD patients and their siblings show that genetic variation in *SORL1* is associated with white matter atrophy and

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hyperintensities, and medial temporal lobe volume in postmortem brain (Cuenco et al., 2008). Recently, it also was shown that the effects of *SORL1* on hippocampal volume may be present healthy young adults (Bralten et al., 2011). Given its role in re-routing amyloid precursor protein (Andersen et al., 2005), downregulation of SORL1 in postmortem brain may be a function of the mechanism of action of *SORL1* genetic risk variants; *SORL1* SNPs have been associated with *SORL1* mRNA expression (McCarthy et al., 2012) and the efficiency of SORL1 translation (Caglayan et al., 2012) in postmortem brain. Although genetic association studies have identified *SORL1* as an AD risk gene, the effect size, like in other non-*APOE* risk genes, is relatively small (Lambert et al., 2013).

In summary, *SORL1* variants are well-established risk factors for AD, though with a weaker influence on risk than *APOE* ε4. Due to its central role in biochemical pathways at the interface of amyloid processing and clearance and vascular metabolism, as well as its upregulation by the growth factor BDNF, SORL1 is likely a hub of convergence for multiple AD risk mechanisms. More work is required to establish where, when, and how *SORL1* confers risk for AD *in vivo*.

1.4.6 Translocator Protein *(TSPO)*

The translocator protein (TSPO, previously known as the peripheral benzodiazepine receptor (PBR)) is a ubiquitous receptor found on the outer mitochondrial membrane. It was first identified in the late 1970s as a high-affinity binding site for the benzodiazepine diazepam (brand name Valium) in rat kidneys (Braestrup & Squires, 1977), but since has been shown to be present in the CNS (Gulyás et al., 2009; Karlstetter et al., 2014). In the healthy brain, TSPO is expressed at low levels in the olfactory bulb, choroid plexus, and glial cells (Banati, 2003; Venneti, Lopresti, & Wiley, 2006). In the AD brain, there is an increase in its expression (M.-K. Chen & Guilarte, 2008) and this increase is thought to be due to the upregulation of TSPO by active microglia (Cagnin et al., 2001; Venneti et al., 2006), which reflect the neuroinflammation associated with AD (Section 1.2.6). PET studies of TSPO radioligands show increased binding in patients with acute brain injury (M.-K. Chen & Guilarte, 2008), multiple sclerosis (Banati et al., 2000; Harberts et al., 2013; Oh et al., 2011), MCI (Yasuno et al., 2012) and AD (Cagnin et al., 2001; Kreisl et al., 2013). TSPO binding is increased in AD subjects, more-so in white matter

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regions (Colasanti et al., 2014; Takano et al., 2013), compared to controls and is correlated with the severity of clinical symptoms (Kreisl et al., 2013).

In 2010, Owen et al. (Owen et al., 2010) investigated a strange phenomenon whereby ~14% of healthy volunteers in neuroinflammation PET imaging studies show no binding signal of the TSPO radioligand [11C]PBR28 (Fujita et al., 2008). By comparing the binding of [11C]PBR28 to that of [3H]PK11195 (a first generation TSPO radioligand that does not show the absent signal phenomenon) in rat and human brain tissue, the authors noticed that each individual fit into one of three classes based on their tissue's preference for binding of [11C]PBR28 over [3H]PK11195; they called these phenotype groups high, medium, and low affinity binders (herein referred to as HABs, MABs, and LABs, respectively). This, along with the observation that TSPO was indeed present in all tissue samples despite the lack of radiographical signal, led to the conclusion that TSPO contains two binding sites, and that differences in TSPO structure may influence binding properties at one of these sites. In 2012, Owen et al. (Owen et al., 2012) demonstrated that the difference between HAB, MAB, and LAB phenotypes could be explained by genotype at a SNP located in exon 4 of the *TSPO* gene, rs6971 (Ala147Thr). Subsequent studies have confirmed that this variant reliably determines the binding affinity of second generation TSPO radioligands in the brain (Mizrahi et al., 2012), where A/A, A/G, and G/G genotypes correspond to HAB, MAB, and LABs. This information is imperative for studies of neuroinflammation using second generation TSPO ligands that show genotype-dependent binding characteristics, as conclusions on receptor density may be strongly biased when rs6971 is not accounted for.

Functionally, it is hypothesized that the Alanine to Threonine substitution at position 147 results in a conformational change in TSPO structure that influences its interaction with a variety of molecules (Korkhov, Sachse, Short, & Tate, 2010; Murail et al., 2008; Owen et al., 2012). This difference in ligand affinity may have important implications for the etiopathology of AD; TSPO ligands have been shown to ameliorate neuroinflammation *in vitro* (Karlstetter et al., 2014), reverse neuropathology and behavioral decline in Alzheimer's disease mouse models (Barron et al., 2013), reduce gamma radiation-induced apoptosis, $A\beta_{42}$-induced neurodegeneration, and premature death in drosophila (R. Lin et al., 2014), as well as confer neuroprotective and regenerative effects *in vivo* and *in vitro* (Ferzaz et al., 2002; Girard et al., 2008; Ryu, Choi, & McLarnon, 2005; Veiga, Azcoitia, & Garcia-Segura, 2005).

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In summary, the *TSPO* rs6971 variant shows a strong effect on the binding of TSPO radioligands in experiments of *in vivo* neuroinflammation. The role of *TSPO* variation in regulating inflammatory AD-related risk mechanisms via its control of TSPO binding properties is not known.

1.4.7 Brain-Derived Neurotrophic Factor *(BDNF)*

The brain-derived neurotrophic factor (BDNF) is a growth factor highly expressed in the healthy hippocampus and it is thought of as a neuroplasticity molecule, important in learning and memory processes (Tapia-Arancibia, Aliaga, Silhol, & Arancibia, 2008). The role of BDNF in neuronal growth and maintenance has made it a clear candidate for association with neurodegenerative disorders, including AD. In 1991, it was shown that BDNF is under-expressed in the hippocampus of patients with AD (Phillips et al., 1991), and since then, both BDNF protein levels (Narisawa-Saito, Wakabayashi, Tsuji, Takahashi, & Nawa, 1996; Tapia-Arancibia et al., 2008) and *BDNF* gene variants (S. E. Harris et al., 2006; Y. Y. Lim et al., 2013; Ventriglia et al., 2002) have been repeatedly associated with AD. One specific *BDNF* gene SNP, known as the Val66Met polymorphism (rs6265), has garnered much attention in the liteature, as it alters the prodomain of proBDNF protein and determines the efficiency with which newly synthesized proBDNF is secreted (Egan et al., 2003). The change in protein sequence influences the efficiency with which proBDNF binds to the intracellular trafficking molecule Sortilin, thereby affecting the levels of secreted protein in an activity-dependent manner. *BDNF* Val66Met has been associated with several AD-related biomarkers including cortical surface area and functional connectivity (C. Wang et al., 2014), brain structure volumes (including hippocampus in healthy subjects (Bueller et al., 2006)) (Hajek, Kopecek, & Höschl, 2012; Toro et al., 2009), cortical thickness and white matter integrity (Voineskos et al., 2011; Yang et al., 2012), and cognitive performance in humans and mouse models (Dincheva, Glatt, & Lee, 2012; Voineskos et al., 2011). In fact, increases in volume of multiple brain regions, including the occipital and medial temporal gyri, have been observed in *BDNF* Val homozygotes as early as weeks after birth (Knickmeyer et al., 2014). Despite this evidence, GWAS of AD have not implicated *BDNF*, and several studies have found no associations of Val66Met with AD risk or AD biomarkers. Many studies have implicated Val66Met in depressive symptoms (Comasco et al., 2011; Dalton,

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Hammen, Najman, & Brennan, 2014; Verhagen et al., 2008), suggesting that the involvement of BDNF may be related to AD-related depression more than AD itself (Borroni et al., 2009). Val66Met may also be important in determining an individual's ability to access cognitive reserve (D. Ward et al., 2015), which implies that effects on known AD pathology and risk phenotypes should not necessarily be directly apparent.

Mechanistically, BDNF is critical for neuronal plasticity and facilitates hippocampal and cortical long-term potentiation (Figurov, Pozzo-Miller, Olafsson, Wang, & Lu, 1996). Learning and memory processes are substantially affected in AD, arising largely from impaired neuronal plasticity (Tapia-Arancibia et al., 2008). In AD patients, BDNF expression is prominently reduced in the hippocampus and the entorhinal cortex (Narisawa-Saito et al., 1996), and these regions are consistently affected in the earliest stages of the disease (Gómez-Isla et al., 1996; J. L. Price et al., 2001). Variation in the *BDNF* Val66Met (rs6265, G>A) polymorphism has been shown to be related to episodic memory performance in younger adults via the hippocampal formation, where methionine (Met) allele carriers had poorer episodic memory performance (Egan et al., 2003). In addition, this polymorphism predicts cognitive performance in elderly individuals (S. E. Harris et al., 2006) and may confer risk for AD (Ventriglia et al., 2002), where Val/Val individuals in these two studies were at risk. The effects of this polymorphism on brain structure and cognition is thought to be a downstream effect of reduced neuronal BDNF secretion, due to altered binding of the Methionine residue in the BDNF pro-domain to chaperone proteins involved in intracellular transport (Z.-Y. Chen et al., 2005; Egan et al., 2003). Recent animal model findings also suggest a compelling potential role for BDNF as a therapeutic agent in AD (Nagahara et al., 2009).

In summary, BDNF is a molecule important in neuroplasticity with a proven relevance to cognitive resilience and brain structure. The *BDNF* Val66Met has been shown to alter the secretion of BDNF in brain; however, the literature on its effects on AD-related biomarkers is heterogeneous.

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1.4.8 Gene-Gene Interaction

William Bateson first coined the term "epistatis" in 1909 (Bateson & Mendel, 1909) to describe masking of the phenotypic expression of one gene by another. The loose term "gene-gene interaction" can be used to refer to epistasis, though as its name implies, epistatic modification effects of one variant on another are nullifying, whereas a gene-gene interaction more broadly may refer to a perfectly antagonistic interaction, whereby the effect of one variant is reversed, rather than nullified, by another. Generally the term "interaction" refers to a departure from independent effects of genetic loci on given phenotypes (Cordell, 2002), though importantly the precise definition of independence is not always clear (i.e. statistical independence is different than biological independence within pathways (reviewed by Wang et al. (Xuefeng Wang, Elston, & Zhu, 2010))). The modeling of genetic interactions can be performed in different ways, producing different results and interpretations. Two common examples are 1) binning groups of subjects by different pairwise combinations of alleles for multiple variants and testing for group differences in a trait, and 2) linear modeling with inclusion of an interaction term and testing the significance of a coefficient describing the conditional effect of one genetic variant on another (Cordell, 2009). In this thesis, gene-gene interaction is defined as the statistical departure from independent effects of SNPs, as determined by the coefficient of the interaction term of a general linear model. This definition allows for the detection of different "types" of interactions as variably defined, including the nullification of a variant's effect (epistasis), as well as the amplification or reversal of a variant's effect, by another.

As mentioned above, the interaction between gene variants is thought to be a potential source of "missing heritability" in AD; linear models used in GWAS (i.e. not including interaction terms) will fail to detect interacting variants that show no marginal effects. Studies exhaustively searching the genome for interaction effects are plagued by multiple comparisons issues (Ueki & Cordell, 2012) and large sample size requirements due to reductions in statistical power (Gauderman, 2002), however, when interactions are present, these types of scans can yield meaningful associations (Marchini, Donnelly, & Cardon, 2005). Using a stepwise approach including standard GWAS followed by pairwise interaction analyses of top SNPs, a landmark study in over 8 000 individuals (Genetic Analysis of Psoriasis Consortium & the Wellcome Trust Case Control Consortium 2 et al., 2010) showed that the effect of one allele (of the endoplasmic reticulum aminopeptdase 1 gene *ERAP1*) only increased risk for psoriasis diagnosis (a complex,

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autoimmune disease) in the presence of a second allele (of human leukocyte antigen C gene *HLA-C*), and the magnitude of this difference was over 15-fold. Importantly, this finding is biologically plausible, as *ERAP1* is involved in the presentation of the class I peptide, which is encoded by the *HLA* gene.

While each of the aforementioned genes has been shown to contribute independently to AD-related biomarkers and both cognitive and postmortem-confirmed diagnoses of AD, there is substantial evidence suggesting that these factors directly interact at the pathway level. SORL1's roles as a receptor for APOE (K Taira, 2001) as well as a modulator of lipoprotein lipase activity (Klinger et al., 2011) place it firmly at the intersection of both amyloidogenic and cerebrovascular risk for AD. This has led experts in the field to suggest that changes in APOE structure due to ε4 genotype could directly influence SORL1's binding to APP, and subsequent risk for disease though Aβ accumulation (Perneczky, Alexopoulos, Eisele, Hans Forstl, & Alexander Kurz, 2010). However, the results of studies analyzing the interaction between *APOE* ε4 and *SORL1* genotype in AD-related phenotypes are mixed, with multiple studies finding no interaction effect on risk for AD (Izzo et al., 2013; Olgiati et al., 2013; Xue, Zhang, Lin, Xu, & Jia, 2014) and few finding a significant interaction for AD pathology (Alexopoulos et al., 2011). Recent *SORL1-APOE* interaction analyses have not found any strong replicable patterns (Yin, Yu, & Tan, 2014), demonstrating the need for further study.

The interaction of *SORL1* with *BDNF*, though less studied, has yielded more convergent findings. Mouse models have shown that SORL1 expression is induced by BDNF, and that the ability of BDNF to lower levels of Aβ levels in primary cortical neurons is dependent on the presence of SORL1 (Rohe et al., 2009). The hypothesis that interacting genetic and non-genetic factors likely account for differences in *SORL1* mRNA expression was first speculated in the original Rogaeva et al. report of *SORL1*'s genetic association with AD (Rogaeva et al., 2007), based on observations that the 3' *SORL1* haplotype only accounted for ~14% of the variance in expression – the authors had insufficient samples to test the SNPs within the 5' region. SORL1 also shares substantial structural homogeneity with another member of the vacuolar protein sorting 10 domain protein (VPS10P) family, Sortilin, which has been shown to bind to the prodomain of BDNF, specifically at the Val/Met substitution site, with varying affinity (Z.-Y. Chen et al., 2005). VPS10P trafficking molecules are important for growth factor transport (perhaps via retromer complex function (Fjorback et al., 2012)), and it has also been shown that

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the cellular response to BDNF signaling is moderated via the trafficking of TrkB by SORL1 (Rohe, Hartl, Fjorback, Klose, & Willnow, 2013). Most recently, work in neurons derived from human induced pluripotent stem cells (iPSCs) showed that the ability of BDNF to up-regulate *SORL1* mRNA expression (first demonstrated by Rohe et al. (Rohe et al., 2009)) was dependent on *SORL1* genotype (Young et al., 2015). This provides strong evidence for the hypothesis in Chapter 6 of this thesis.

1.4.9 Imaging-Genetics

In 2000, three seminal reports of differences in brain imaging phenotypes due to genetic variation (In order of publication date: SPECT-*SLC6A3* (Heinz et al., 2000), PET-*APOE* (G. W. Small et al., 2000), and functional MRI (fMRI)-*APOE* (Bookheimer et al., 2000)) effectively founded the field of imaging-genetics. As defined (Hariri, Drabant, & Weinberger, 2006; Hariri & Weinberger, 2003), the imaging-genetics approach capitalizes on the fact that brain structural and functional phenotypes lie closer to the biological substrates of complex behavior and cognition that constitute clinical diagnosis (i.e. they are intermediate phenotypes (Meyer-Lindenberg & Weinberger, 2006)), and thus offer improved association and penetration when analyzed from a genetic standpoint. Further, the use of non-invasive brain imaging *in vivo* allows for some interpretation regarding region-specific mechanisms of brain susceptibility and resilience that are altered by genetics. This information cannot be inferred as well from measures of cognition or pathology alone.

There are two main types of imaging-genetics approaches (Verhoeven, Tuinier, & van der Burgt, 2008): one in which a gene implicated in disease is assessed for its contribution to imaging phenotypes (so-called bottom-up approach, analogous to reverse genetics), and one in which the imaging phenotype that is known to be affected in disease is used as an outcome for discovery of disease-related genes, as in a complex trait GWAS design (top-down approach, analogous to forward genetics). Much work from our group has successfully used the former approach to characterize known risk variants for AD (Voineskos et al., 2011) and Schizophrenia (Lett et al., 2013) across the lifespan using multi-modal imaging phenotypes. Work by the ENIGMA consortium and others have produced large GWAS analyses of brain imaging phenotypes, including those for hippocampal volume, intracranial volume and other subcortical structure

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volumes (Hibar et al., 2015; Stein et al., 2012), cortical thickness (Furney et al., 2011), and white matter microstructure (Jahanshad, Kochunov, et al., 2013), though genes implicated across studies are different and wide- ranging in putative or known functions (e.g. *TESC*, *HMGA2*, *DDR3*, *ZNF292*, *ARPP-21*). These top-down GWAS approaches suffer from the same issues as diagnostic GWAS, except that the problem of multiple comparisons is often compounded based on the high-dimensionality of imaging data, and typically genetic effects are very small (e.g. the top SNP from Stein et al. (Stein et al., 2012) explained ~0.27% of hippocampal volume variance) (Medland, Jahanshad, Neale, & Thompson, 2014).

The initial study of MRI-based phenotypes with *APOE* variation (Bookheimer et al., 2000), published in the New England Journal of Medicine, examined 30 adults with normal cognition (16 *APOE* ε4 carriers, and 14 *APOE* ε4 non-carriers) using fMRI, finding that ε4 carriers showed increases in both the strength and topological extent of brain activation (measured using the brain-oxygen-level dependent (BOLD) signal (Logothetis & Wandell, 2004)) during a verbal memory task. This supports the hypothesis that ε4 carriers, who are at increased risk for AD, must employ more widespread compensatory brain networks and exert additional cognitive work to accomplish the same memory task as non-ε4 carriers. Since this report, dozens of others have analyzed BOLD signal between *APOE* genotype groups, with mixed results (reviewed by Trachtenberg et al. (Trachtenberg, Filippini, & Mackay, 2012)): 37% report increases, 26% report decreases, 19% report both increases and decreased, and another 19% report no significant changes in activation due to ε4 carriage. Potential reasons for this heterogeneity include inter-study differences in protocol, age interactions across the lifespan (most studies included only mid- to late-life samples), as well as well as substantial inter-individual differences in BOLD activation that are observed for certain cognitive tasks (Plichta et al., 2012). Structural imaging biomarkers including volumetry, cortical thickness, and especially DTI (outlined in Section 1.3.5 and 1.3.6), may represent more robust, trait-reflective imaging measures by which the effect of genetics can be accurately measured (Buchanan, Pernet, Gorgolewski, Storkey, & Bastin, 2014; Duchesne, Valdivia, Mouiha, & Robitaille, 2012; Maclaren, Han, Vos, Fischbein, & Bammer, 2014; Zhao et al., 2015).

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1.5 Outline of Experiments

Genetic investigations have the potential to identify molecular subtypes of individuals at any stage of the lifespan simply and non-invasively. GWAS and candidate studies together have pointed toward a diverse set of biochemical pathways and loci, raising questions about their relative contributions to AD susceptibility and progression. Answering these questions will inform which biomarkers should be used in intervention studies for genetically at-risk subgroups, and the time-point in the lifespan at which such a prevention/treatment study should be started.

The next chapter (2) will introduce each of the experiments performed as part of this thesis (contained in Chapters 3-6) and outline each of their central hypotheses. Chapters three and four have been published in two high impact, peer-reviewed journals; JAMA Psychiatry (Letter to the Editor) and Molecular Psychiatry (Original Article), respectively. Chapter five has been accepted for publication in the Journal of Cerebral Blood Flow and Metabolism (Negative Report), and Chapter six is currently in the process of submission.

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Chapter 2

2 Overview of Experiments, and Hypotheses

2.1 *APOE* ε4, Aging, and Effects on White Matter across the Lifespan

2.1.1 Background

Much work has been done characterizing the mechanisms of risk posed by the APOE4 isoform across the human lifespan. Seminal work in children and adolescents has demonstrated that the ε4 allele may exert significant effects on the thickness of entorhinal cortex from an early age (Shaw et al., 2007), but studies are not in agreement over whether or not genotype influences rate of cortical thinning longitudinally (Espeseth et al., 2008). In 2012, a study by Nichols et al. (Nichols et al., 2012) analyzed the effect of *APOE* ε4 carrier status on the activation of hippocampus during an episodic memory task using fMRI. Interestingly, they found that the interaction was dependent on the age of the healthy participants; ε4 carriers showed no significant decline in hippocampal recruitment across the age range (19-77 years) whereas the ε3 homozygotes showed steady decline with age (Nichols et al., 2012). This observation suggested that younger ε4 carriers, who are known to be at higher risk for AD, showed greater hippocampal function than the neutral risk ε3 homozygotes. We therefore designed an experiment to determine if this *APOE* ε4 age-dependent effect on hippocampal engagement may be driven by structural connectivity between hippocampus and cortex, by measuring microstructural integrity of a white matter tract called the cingulum bundle, using DTI.

2.1.2 Hypothesis

We hypothesized that we would observe an age-by-genotype interaction for *APOE* ε4 carriers vs. non-carriers, whereby the *APOE* ε4 carriers would show a stronger decline in FA with increasing age, cross-sectionally, compared to the ε4 non-carriers. This would provide a potential neuroanatomical explanation for the functional interaction observed by Nichols et al. (Nichols et al., 2012).

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2.2 The *SORL1* Gene and Convergent Neural Risk for Alzheimer's Disease across the Human Lifespan

2.2.1 Background

Following growing evidence for the involvement of *SORL1* variants in determining risk for AD (Rogaeva et al., 2007), Cuenco et al. (Cuenco et al., 2008) sought to investigate the effects of these variants on cerebral and hippocampal volume, as well as white matter hyperintensity burden. They found that alleles of risk genes in the 5' region of *SORL1* that had been associated with risk for AD were actually protective in regard to white matter changes that are typically indicative of AD risk. This suggested a divergence in the nature of genetic risk conferred by the same *SORL1* variants, supported by a 2011 study (Bralten et al., 2011) showing that the same brain-protective, yet diagnostic-risk alleles were also associated with increased hippocampal volumes in young healthy adults. In order to better understand the pattern of *SORL1*'s effects on brain structure as they relate to AD risk in healthy subjects, a more informative intermediate phenotype that is predictive of the earliest phases of AD would have to be used. To this end, it was shown by Zhuang et al. (Zhuang et al., 2012) that individuals who converted from cognitively normal to a state of a-MCI over a two year period showed substantial changes in FA of the precuneus, parahippocampal cingulum, parahippocampal gyrus, and fornix. We therefore designed an experiment to examine the effects of *SORL1* on white matter FA across the lifespan. To make our analyses more definitive, we included a second imaging-genetics sample (spanning early life, ages 8-40) for replication. To further confirm a potential mechanism of action consistent with *SORL1*'s role in amyloidogenic processing, we examined *SORL1* effects on gene expression (again, across the human lifespan) as well as postmortem amyloid neuropathology in a very large cohort of healthy, MCI, and AD subjects (the ROS/MAP combined sample).

2.2.2 Hypothesis

We hypothesized that AD risk variants within two haplotype blocks of *SORL1* would be associated with a) lower fractional anisotropy in specific white matter tracts (primarily those implicated in AD and the conversion from CN to amnestic MCI: cingulum bundle, superior

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longidunial fasciculus, and corpus callosum), b) deficits in *SORL1* mRNA expression, and c) increases in late-life amyloid neuropathology. For parts a) and b), where lifespan data were available, we hypothesized that the effects of genotype may be exerted early in life, as the independent mechanism of SORL1-dependent APP recycling is thought to be active throughout life and not dependent on other aging factors (such as accumulating vascular or neurotoxic insults).

2.3 Cerebrovascular and Microglial States are Not Altered by Functional Neuroinflammatory Gene Variant

2.3.1 Background

In line with the neuroinflammatory hypothesis of AD, it has been shown that levels of activated microglia are increased in patients compared to controls. This microglial activation can be measured *in vivo* using PET radioligands with binding specificity to TSPO, which is highly upregulated in active microglia (Cagnin et al., 2001; Venneti et al., 2006). The *TSPO* rs6971 polymorphism has been established as a strong modifier of TSPO protein binding properties to multiple radioligands (Owen et al., 2012), however the possibility that this altered binding to exogenous ligands would also have implications for molecular interactions with endogenous ligands seems to have gone largely unaddressed. Recently, it was shown that rs6971 had no effects on Aβ deposition (measured using [18F]Flobetapir PET) or cognition in the ADNI cohort (Fan et al., 2015). While Fan et al. may have chosen these outcome measures due to their relevance to AD risk, the functions of TSPO as a cholesterol transporter (Taylor, Allen, & Graham, 2014) and putative regulator of inflammatory response (Bae, Shim, Balu, Kim, & Yu, 2014; Karlstetter et al., 2014; M. Wang et al., 2014) suggest that perhaps phenotypes more specific to cerebrovascular and inflammatory AD risk factors might be impacted to a greater, potentially detectable degree. We therefore designed a study to examine the effects of the functional *TSPO* rs6971 variants on relevant cerebrovascular and inflammatory phenotypes including macro and micro cerebral infarcts, white matter hyperintensity burden, blood-based inflammatory biomarkers, and microglial activation measured postmortem.

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2.3.2 Hypothesis

We hypothesized that individuals would show *TSPO* rs6971 genotype-dependent changes in white matter hyperintensity volume, likelihood of having cerebral infarcts (both micro and macro), levels of pro-inflammatory plasma biomarkers, as well as microglial activation. Due to the anti-inflammatory action of certain TSPO ligands, we hypothesize that that low-affinity binding groups would have exacerbated pathology and increased levels of pro-inflammatory biomarkers vs. medium- and high-affinity binding groups, as determined by genotype.

2.4 Genetic Interaction between *SORL1* and *BDNF* Regulates Isoform-Specific *SORL1* Expression and Brain Amyloid

2.4.1 Background

SORL1, like any other eukaryotic exon-containing gene, can undergo the process of alternative splicing to generate transcriptomic diversity in the cell. Despite the importance of this process in AD (Tazi, Bakkour, & Stamm, 2009), very few studies have analyzed the differential expression of *SORL1* transcripts either in AD or as a result of genotype differences. Given the role of SORL1 in APP processing and Aβ lysosomal targeting, the regulation of its expression is of great interest. Importantly, different types of *SORL1* transcripts, which may be lacking important protein binding domains that allow SORL1 to carry out its functions, can be quantified using RNA-sequencing technology. Recent work by Young et al. (Young et al., 2015), demonstrated that the effect of BDNF on *SORL1* expression was dependent upon *SORL1* genotype. This study immediately raises questions about the relationship between functional gene variants in both genes: since levels of BDNF secretion in the brain are altered by the Val66Met polymorphism (Egan et al., 2003), then it follows that we might observe effects of the Val66Met polymorphism on *SORL1* expression that are dependent on *SORL1* genotype. Further, as indicated by findings that BDNF's effects on amyloid are modulated by SORL1, the question of whether or not any gene regulatory effects of a *BDNF-SORL1* interaction would concurrently influence amyloid pathology should be addressed. We designed a study to test these questions by analyzing all SNPs within the SORL1 gene for interaction with Val66Met in models predicting *SORL1* expression of 13 isoforms in the ROS/MAP cohort. We followed this up with analyses of

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amyloid plaque deposition in the same postmortem sample as well as of *in vivo* amyloid binding using [18F]Florbetapir PET in the ADNI sample. To test for any brain structural effects of the *SORL1-BDNF* interaction, we analyzed over 1 300 MRI and DTI scans from ADNI and ROS/MAP.

2.4.2 Hypothesis

We hypothesized that *SORL1* mRNA expression would be dependent on the interaction of the *BDNF* Val66Met polymorphism and common variants within the 5' region of *SORL1*. Using RNA-seq data, we were able to test this at the level of multiple *SORL1* transcripts. Given SORL1's role in amyloidogenic processing, we further hypothesized that any resultant changes in *SORL1* expression may influence amyloid levels postmortem and *in vivo* (as measured using [18F]Florbetapir PET). If genetic interactions were affecting gene expression and amyloid, then we might expect to see parallel changes in brain structure (both gray and white matter) that are especially vulnerable AD. From this, our last hypothesis stated that genotypic groups showing altered *SORL1* mRNA expression and amyloid levels would also show differences in entorhinal cortex volume and white matter tract microstructural integrity (measured using DTI).

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Chapter 3

3 *APOE* ε4, Aging, and Effects on White Matter across the Adult Life Span

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A link to the published paper can be found at:

http://archpsyc.jamanetwork.com/article.aspx?articleid=1695575

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3.1 Letter to the Editor

The recent paper by Nichols et al. (Nichols et al., 2012) is an important and useful addition in efforts to understand the effects of *APOE* ε4 status on brain function. It was the first to examine the effects of apolipoprotein ε4 status using an episodic memory task during functional magnetic resonance imaging (MRI) across the adult lifespan. During task performance, the authors found an age by genotype interaction, whereby younger ε4 carrier participants showed less hippocampal activation compared with ε4 non-carriers, and older ε4 participants trended toward increased activation compared to older ε4 non-carriers. Since the original fMRI finding by Bookheimer et al.(Bookheimer et al., 2000), several studies have examined neural effects of *APOE* ε4 status during episodic memory performance. A recent review (Trachtenberg et al., 2012) highlighted the almost equal distribution of either 'under' or 'over' activation in ε4 carriers compared to noncarriers during episodic memory fMRI studies in both younger and older participants. Therefore, despite the results of Nichols et al., some open questions remain. One important consideration (or confound) of ε4 activity on the BOLD signal is the well-documented effect of the ε4 allele on resting cerebral blood flow (Trachtenberg et al., 2012). Indeed, Alzheimer's patients early in the disease process show decreased blood flow at rest in the hippocampal complex and posterior cingulate cortex. Furthermore, the ε4 allele has been shown to influence functional connectivity at-rest among regions that comprise episodic memory circuitry (E. T. Westlye, Lundervold, Rootwelt, Lundervold, & Westlye, 2011).

The cingulum bundle, a white matter tract that serves as the main anatomical connection from hippocampus to posterior cingulate cortex, and other cortical midline regions, may provide a useful framework toward understanding conflicting results in task-based ε4 studies. Cingulum bundle integrity is highly correlated with hippocampal atrophy and represents the major source of functional disconnection between hippocampus and posterior cingulate cortex in early AD (Chételat et al., 2003). Deterioriation of this structure predicts conversion of cognitively normal amnestic MCI converters, and is predictive of subsequent episodic memory decline (Zhuang et al., 2012). Brain deafferentation through the cingulum plays a substantive role in progressive development of cognitive impairment in AD (Bozzali et al., 2012). While others have examined the relationship between ε4 status and cingulum bundle integrity, none have done so across the adult lifespan. In 97 right-handed healthy adults (age range 18-86 years, 52 males, 45 females)

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using diffusion tensor imaging methods previously described (Voineskos et al., 2009), we report an age by ε4 carrier interaction predicting microstructural integrity of the cingulum bundle ($F_{4,92}$=8.2, p=0.005) (Figure 3-1). E4 carrier (n=27) and ε4 non-carrier (n=70) groups were not different on sex, age or IQ. Our findings parallel those of Nichols et al. (Nichols et al., 2012): older ε4 carriers (>50 years of age) demonstrated reduced microstructural integrity compared to ε4 non-carriers ($F_{2,42}$=3.9, p=0.05), while the opposite finding was made in young ε4 carriers ($F_{2,37}$=5.4, p=0.03). Others have examined ε4 status effects on white matter integrity in discrete age groups (Heise, Filippini, Ebmeier, & Mackay, 2011), but none have used an adult lifespan approach.

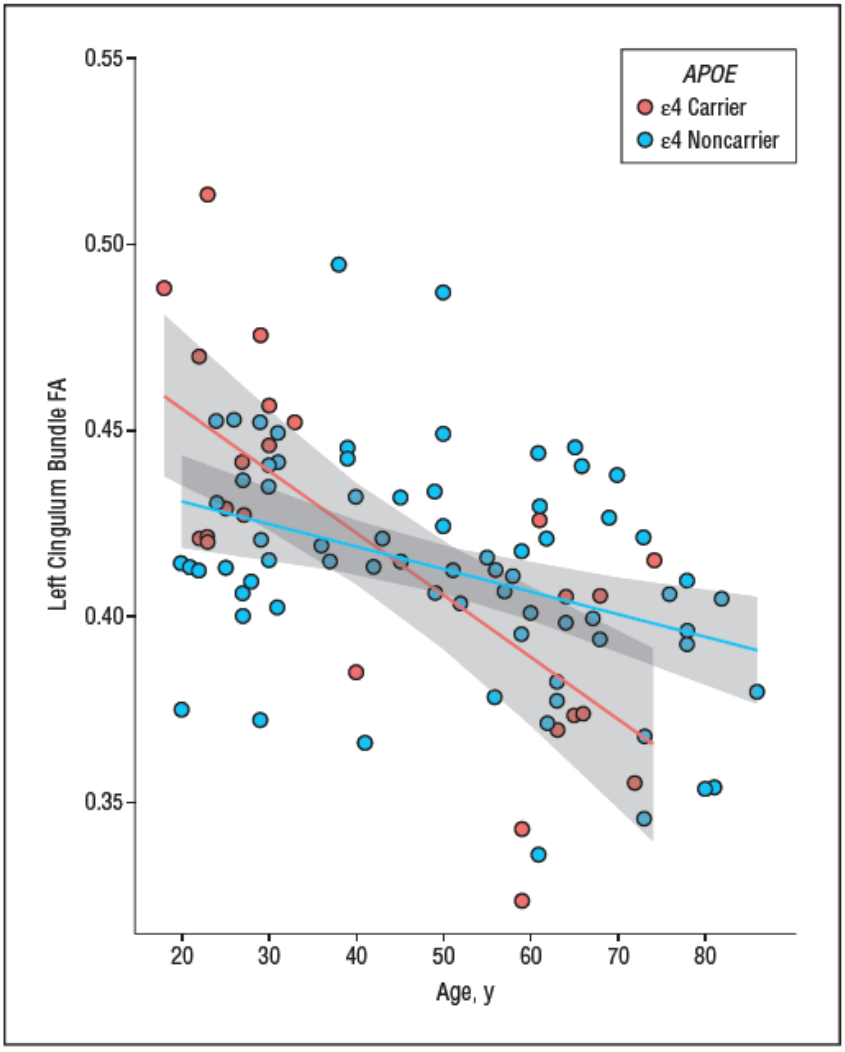

Figure 3-1. Demonstration of *APOE* ε4 allele carrier status x age interaction predicting fractional anisotropy (FA) of the left cingulum bundle.

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Our results may provide an explanatory mechanism for the heterogeneous pattern of functional activation during episodic memory tasks in ε4 carriers. At rest, disruption of the cingulum bundle predicts substantial blood flow alterations in hippocampus and posterior cingulate cortex. Furthermore, when a structure such as the hippocampus (itself vulnerable in ε4 carriers) is engaged during an episodic memory task, disruption of a major line of anatomical communication such as the cingulum bundle may lead to compensatory over or under activation. Our results also support the concept of 'anatagonistic pleiotropy' (Ihle, Bunce, & Kliegel, 2012), whereby young ε4 carriers appear to have higher FA compared to ε4 non-carriers, consistent with studies showing that younger ε4 carriers may perform better on cognitive tasks compared to ε4 non-carriers at this stage of the lifespan. When taken together, the study by Nichols et al., and our own data, support the use of a lifespan approach to help identify genetically-based timing and direction of neural risk for dementia. In the future, studies of APOE binding partners, and the use of brain network phenotypes, may broaden our understanding of this fascinating, yet still perplexing, age by genotype interaction.

3.2 Acknowledgements

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Chapter 4

4 The *SORL1* Gene and Convergent Neural Risk for Alzheimer's Disease across the Adult Lifespan

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4.1 Abstract

Prior to intervention trials in individuals genetically at-risk for late-onset Alzheimer's disease, critical first steps are identifying where (neuroanatomic effects), when (timepoint in the lifespan) and how (gene expression and neuropathology) Alzheimer's risk genes impact the brain. We hypothesized that variants in the sortilin-like receptor (*SORL1*) gene will affect multiple Alzheimer's phenotypes before the clinical onset of symptoms. Four independent samples were analyzed to determine effects of *SORL1* genetic risk variants across the lifespan at multiple phenotypic levels: 1) microstructural integrity of white matter using diffusion tensor imaging in two healthy control datasets (n=118, age 18-86, and n=68, age 8-40); 2) gene expression using the BrainCloud postmortem healthy control sample (n=269, age 0-92); and 3) Alzheimer's neuropathology (amyloid plaques and tau tangles) using a postmortem sample of healthy, mild cognitive impairment (MCI), and Alzheimer's individuals (n=710, age 66-108). *SORL1* risk variants predicted lower white matter fractional anisotropy in an age-independent manner, in fronto-temporal white matter tracts in both samples at 5% FWE-corrected thresholds. *SORL1* risk variants also predicted decreased *SORL1* mRNA expression, most prominently during childhood and adolescence, and significantly predicted increases in amyloid pathology in postmortem brain. *SORL1* Alzheimer's risk variants predicted impairment in white matter pathways and reduced expression of *SORL1* mRNA during neurodevelopmental phases of the human lifespan. Further, the neuropathological mechanism of risk appears to primarily involve amyloidogenic pathways. Interventions targeted toward the SORL1 amyloid risk pathway may be of greatest value during early phases of the lifespan.

4.2 Introduction

Late-onset Alzheimer's disease (AD) (i.e. onset after 65 years of age) is the most common form of dementia and is expected to affect over 115 million individuals worldwide by 2050 (Prince & Jackson, 2009). However, there is accumulating evidence that subtle deterioration of brain structure may be present decades before the late-life emergence of clinical signs and symptoms in people genetically at-risk for this disorder (Fox, 2012; Reiman et al., 2012). The failure of phase 3 trials in early stages of AD has hastened calls for intervention prior to clinical disease

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onset in genetically at-risk groups in whom effects on brain structure or function might be present (Reiman et al., 2010). These brain alterations, detectable using advanced neuroimaging approaches, can then serve as markers of treatment efficacy during clinical trials. However, prior to the initiation of such trials, systematic investigation of where (neuroanatomic effects), when (timepoint in the lifespan) and how (gene expression and neuropathology) AD risk genes impact the brain is required. After the *APOE* gene, which has not always shown consistent neural effects prior to disease onset (Felsky & Voineskos, 2013; Trachtenberg et al., 2012), a small number of confirmed risk genes (Bertram & Tanzi, 2012) for late-onset AD require such systematic investigation.

Among these risk genes is the sortilin-related receptor, L(DLR class), A repeats containing (SORL1, sorLA, LR11) gene, which codes for an ApoE receptor (Hoe & Rebeck, 2008). SORL1 is thought to act within classical AD risk pathways by helping direct the preferential transport of amyloid precursor protein (APP) to endosomal recycling pathways, away from beta-secretase cleavage and subsequent beta-amyloid (1-42) ($A\beta_{42}$) formation (Andersen et al., 2005; Offe et al., 2006). Disruption of SORL1 has also been shown to influence tau-related cellular processes (Capsoni et al., 2013). Furthermore, SORL1 operates at the interface of AD and vascular disease risk by acting in cerebrovascular disease pathways related to AD, where it plays a central role in lipoprotein lipase trafficking (Klinger et al., 2011).

SORL1 genetic variants have been associated with risk for AD in several ethnic groups (J. H. Lee, Barral, & Reitz, 2008; Reitz et al., 2011; Rogaeva et al., 2007). These studies have implicated single nucleotide polymorphisms (SNPs), primarily within two haplotype blocks at the 5' and 3' ends of the gene. Recently identified mutations at both ends of the SORL1 gene have been described in early-onset AD (Pottier et al., 2012), suggesting a potentially causative role for this gene. *SORL1* risk variants have been associated with *SORL1* expression in postmortem brain (Caglayan et al., 2012; Grear et al., 2009), and down-regulation of SORL1 in AD and mild cognitive impairment (MCI) brain has also been shown (Sager et al., 2007; Scherzer et al., 2004). Furthermore, these variants have been associated with white matter atrophy and hyperintensities in late-life (Cuenco et al., 2008), as well as hippocampal volume in early adult life (Bralten et al., 2011). However, white matter microstructure (i.e. fractional anisotropy) was recently identified as the best MRI-based predictor of conversion from normal

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cognitive state to amnestic cognitive impairment (Zhuang et al., 2012), underscoring the potential of this neuroimaging phenotype to improve detection of early risk for late-onset AD.

In four independently collected samples, we assessed the effects of *SORL1* risk variants on gene expression, AD neuropathology, and white matter microstructure *in vivo*, using the added dimension of a lifespan approach. We hypothesized that *SORL1* risk variants would influence white matter microstructure and *SORL1* gene expression, in a temporally linked manner, decades prior to the timeframe of typical AD-onset. Given the putative effect of decreased *SORL1* expression on the APP pathway, we also hypothesized that *SORL1* risk variants would predict increased amyloid-β plaque levels in postmortem brain.

4.3 Methods

4.3.1 Neuroimaging (CAMH and Zucker Hillside Samples)

4.3.1.1 CAMH Sample

142 healthy volunteers (age 18-85) were recruited at the Centre for Addiction and Mental Health (Toronto, Canada). All individuals met the following criteria for eligibility in this study: negative urine toxicology at time of recruitment, no history of substance abuse, head injury with loss of consciousness, seizure, or other neurological disorders, as well as no first degree relative with diagnosis of psychotic mental illness. All study participants were interviewed by a psychiatrist, and assessed with the Structured Clinical Interview for DSM-IV Disorders (First, Gibbon, & Williams, 1995) to rule out the presence of any psychiatric disorder. Of 142 total subjects recruited, 119 completed full screening, imaging and genotyping protocols successfully (102 Caucasian, 10 Asian, 6 other). Genotypic groups were matched for socio-demographic factors (see Table 4-1). The protocol was approved by the local Research Ethics Board, and all participants provided informed, written consent. All participants were genotyped for six single nucleotide polymorphisms (SNPs) in the SORL1 gene (sequentially numbered SNPs 8-10 and 23-25 as defined by Rogaeva and colleagues, see Table 4-2) (Rogaeva et al., 2007) and two in *APOE* (rs429358 and rs7412) using previously published methods (Felsky et al., 2012). Genotype calls were made manually, with two laboratory personnel independently verifying results. 10% of sample genotypes underwent quality control duplication. Genotyping success

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rates of 100% and 98.4% were achieved for all *SORL1* loci and both *APOE* loci, respectfully. In the CAMH sample, the 5' *SORL1* SNPs (8-10) were found to be in near-perfect LD, with a single individual possessing a non-conforming haplotype. This individual was excluded from analysis due to the rarity of this haplotype in our sample, resulting in a final n=118. This final subset of subjects also completed an extensive cognitive battery, which included a measure of verbal episodic memory from the Repeatable Battery for the Assessment of Neuropsychological Status (RBANS) (Kevin Duff et al., 2008), and a measure of executive function using the Trails A and B tests. DTI was conducted on a 1.5T GE Echospeed scanner (General Electric, Milwaukee, WI). A single-shot spin echo planar sequence was used with diffusion gradients applied in 23 noncollinear directions and $B = 1000$ s/mm^2. Two $B = 0$ images were obtained. Fifty-seven sections were acquired for whole brain coverage oblique to the axial plane. Voxels were 2.6 mm isotropic. The field of view was 330 mm, and the size of the acquisition matrix was 128 × 128 mm, with an echo time of 85.5 milliseconds and a repetition time of 15 000 milliseconds. The entire sequence was repeated 3 times to improve signal to noise ratio.

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Table 4-1. Demographic Summary Statistics by SORL1 rs689021 Genotype, Recessive Model

	CAMH Sample (n=118)			Zucker Hillside Sample (n=68)		
	SORL1 rs689021 Genotypic Groups		Diff	SORL1 rs689021 Genotypic Groups		Diff
Demographic	G-carriers (n=95)	A/A (n=23)	*p*	G-carriers (n=53)	A/A (n=15)	*p*
Both Samples						
Age, Y(SD)	45(19)	44(19)	0.87	22(7)	21(8)	0.68
Education, Y(SD)	15(2)	15(2)	0.69	13(4)	11(4)	0.15
IQ (SD)	118(8)	118(9)	0.92	107(10)	106(8)	0.89
Sex	52 M, 43 F	15 M, 8 F	0.48	25 M, 28 F	8 M, 7 F	0.77
Handedness	86 R, 6 L, 3 A	23 R	0.64	59 R, 4 L	13 R, 2 L	0.59
Ethnicity	84 Cau, 7 As, 4 O	18 Cau, 3 As, 2 O	0.32	53 Cau	15 Cau	-
APOE ε4, N(%)	24(25)	6(26)	1	9(17)	2(13)	1
CAMH Only						
MMSE (SD)	29(1)	29(1)	0.76	-	-	-
BMI (SD)	25(5)	26(4)	0.48	-	-	-
Systolic BP (SD)	124(16)	125(16)	0.80	-	-	-
Diastolic BP (SD)	76(11)	74(8)	0.25	-	-	-
CIRS-G (SD)	0.9(0.6)	1.1(0.7)	0.14	-	-	-

Note: IQ measured using standardized scores of the Weschler Test of Adult Reading (WTAR) for the CAMH sample and the Wide Range Achievement Test 3 (WRAT3) for the Zucker Hillside sample. Continuous variables (age, education, BMI, IQ, MMSE, BP, and CIRS-G) were analyzed for genotypic group differences using a student's t-test (two-tailed). Factor variables (sex, handedness, ethnicity, and APOE ε4 status) were analyzed using Fisher's exact test (two-tailed). Y = years; M = male; F = female; R = right; L = left; A = ambidextrous; Cau = Caucasian; As = Asian; O = other; MMSE = Mini Mental Status Exam; BMI = body mass index (height(cm)/weight(kg)^2); BP = blood pressure; CIRS-G = Cumulative Illness Rating Scale – Geriatrics.

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Table 4-2. Details for Analyzed SNPs in SORL1 5' Haplotype by Study Sample

Sample	*SORL1* SNPs Directly Genotyped or Imputed (D/I)						
	SNP	**#**	**Location (Chr:Pos)**	**Orientation/Strand**	**Alleles (min/ maj)**	**Phenotypes**	**# Independent Tests Performed**
CAMH	rs668387 (D)	8	11 : 121367921	Rev/B	T/C	White Matter FA	1 (haplotype in perfect LD, dominant and recessive models)
	rs689021 (D)	9	11 : 121371120	Rev/T	A/G		
	rs641120 (D)	10	11 : 121380965	Fwd/B	T/C		
Zucker Hillside	rs668387 (I)	8	11 : 121367921	Rev/B	T/C	White Matter FA	1 (haplotype in perfect LD, dominant and recessive models)
	rs689021 (I)	9	11 : 121371120	Rev/T	A/G		
BrainCloud	rs689021 (D)	9	11 : 121371120	Rev/T	A/G	SORL1 mRNA	1 (recessive model)
ROS/MAP	rs668387 (I)	8	11 : 121367921	Rev/B	T/C	Aβ Plaques	3 (one in each diagnostic group: HC, MCI, AD)
	rs689021 (I)	9	11 : 121371120	Rev/T	A/G		
	rs641120 (I)	10	11 : 121380965	Fwd/B	T/C	PHFtau Tangles	3 (one in each diagnostic group: HC, MCI, AD)

Note: SNP locations according to NCBI dbSNP build 37. SNP #s correspond to those assigned by Rogaeva et al. (2007). CAMH = Centre for Addiction and Mental Health; ROS/MAP = Religious Orders Study / Memory and Aging Project; D = directly genotyped; I = imputed (see Methods section); Chr = chromosome; Pos = position; Rev = reverse direction; Fwd = forward direction; B = bottom strand; T = top strand; Ex. = exonic; syn = synonymous; Ala = alanine; min = minor allele; maj = major allele; FA = fractional anisotropy; LD = linkage disequilibrium; mRNA = messenger ribonucleic acid; Aβ = beta-amyloid; PHFtau = paired helical filament tau; HC = healthy controls; MCI = mild cognitive impairment; AD = Alzheimer's disease. Genotype frequencies of compared groups are reported for each sample separately in Tables 4-1 and 4-3.

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4.3.1.2 Zucker Hillside Sample

To better characterize the effects of *SORL1* during white matter development which plateaus in the 4th decade of life (L. T. Westlye et al., 2010), 68 healthy Caucasian subjects (age 8-40) were examined from an ongoing study at the Zucker Hillside Hospital, Glen Oaks, NY, by advertisement and word of mouth. Exclusion criteria included serious medical illness and any history of psychosis or major mood disorders, as determined by structured and semistructured assessments (First, Spitzer, Gibbon, & Williams, 2002; Kaufman et al., 1997; Peters et al., 2012). Genotypic groups were matched for socio-demographic factors (see Table 4-1). Further details on sample characteristics, inclusion and exclusion criteria have been previously published (Peters et al., 2012). Genotyping for all subjects was performed using the Illumina (San Diego, CA, USA) HumanOmniExpress-12v1.0 BeadChips assay, which contained information for SNP 8 and SNP 9 (see Table 4-2). Missing genotypes were imputed using data from HapMap 3. *APOE* ε4 status was derived from rs4420638 (a proxy for *APOE* rs429358, where the rs4420638 G allele is linked to ε4). All subjects received a DTI exam at the North Shore University Medical Center, Manhasset, NY, on a GE Signa HDx 3.0T system (General Electric, Milwaukee, Wisconsin). The sequence included volumes with diffusion gradients applied along 31 non-parallel directions (b = 1000 s/mm2) and 5 volumes without diffusion weighting (TR = 14 s, TE = min, matrix = 128 x 128, FOV = 240 mm). Each volume consisted of 51 contiguous 2.5-mm axial slices acquired parallel to the anterior-posterior (AC-PC) commissural line using a ramp sampled, double spin-echo, single shot echo-planar imaging (EPI) method. Data acquisition used parallel imaging with an acceleration factor of 2.

4.3.2 Postmortem SORL1 mRNA (BrainCloud Sample)

The BrainCloud postmortem dataset consists of 269 human subjects, ranging from fetal to late-life, each with genomic data and transcriptomic data for the prefrontal cortex. All subjects had no history of significant psychological problems or psychological care, psychiatric admissions, or drug detoxification and no known history of psychiatric symptoms or substance abuse, as determined by both telephone screening and medical examiner documentation, as previously described (Lipska et al., 2006). All individuals from the BrainCloud dataset were genotyped using either Illumina (San Diego, CA, USA) Infinium II 650K or Illumina Infinium HD Gemini

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1M Duo BeadChips and mRNA quantified with the Illumina Human 49K Oligo array (HEEBO-7 set) according to previously published methods (Colantuoni et al., 2011).

4.3.3 Postmortem Amyloid Load and Tangles (Religious Orders Study {ROS} and Memory and Aging Project {MAP} Sample)

Participants from ROS are older nuns, priests and brothers from across the US (Bennett, Schneider, Arvanitakis, et al., 2012), and those from MAP are residents of approximately 40 senior housing facilities in the Chicago metropolitan area, including subsidized housing facilities, retirement communities, and retirement homes as previously described (Bennett, Schneider, Buchman, et al., 2012). Both studies, approved by the Institutional Review Board of Rush University Medical Center, enroll older persons without dementia who agree to annual evaluation and autopsy. All subjects were assessed with a comprehensive decision tree algorithm as well as a uniform, structured, clinical evaluation that included a self-report medical history obtained by trained nurses and research technicians, a neurologic examination by trained nurses and cognitive function testing by trained neuropsychological test technicians. Please see Bennet et al. (Bennett et al., 2006) for further detail. The follow-up rate exceeds 95% and the autopsy rate exceeds 90%. At the time of analysis, genomic data were available from n=710 autopsied subjects in total (249 CN, 182 MCI, 279 AD). For regional quantification of Amyloid-β plaques and paired helical filament tau (PHFtau) tangles in postmortem brains, tissue blocks were analyzed from entorhinal cortex proper, hippocampus (CA1/subiculum), superior frontal cortex, dorsolateral prefrontal cortex, inferior temporal cortex, angular gyrus cortex, anterior cingulate cortex, and calcarine cortex. Immunohistochemical analysis was performed to quantify Amyloid-β and PHFtau for an average measure of pathology across all regions. Details of autopsy procedure and quantification of neuropathological measures have been previously published (Bennett, Wilson, Boyle, Buchman, & Schneider, 2012). Genomic data was generated using the Affymetrix (Santa Clara, CA, USA) Genechip 6.0 platform, with *APOE* and *SORL1* SNP 8-10 genotypes imputed from MACH (version 1.0.16a) and HapMap release 22 CEU (build 36), as previously published (see Table 4-2) (Chibnik et al., 2011). Genotype groups were matched for socio-demographic characteristics as described in Table 4-3.

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Table 4-3. ROS/MAP Demographic Summary Statistics by *SORL1* rs689021 Genotype, Recessive Model

	ROS/MAP Postmortem Sample (n=705)								
	SORL1 rs689021 Genotypic Groups by Diagnosis								
Demographic	CN (n=247)			MCI (n=180)			AD (n=278)		
	G-car (n=201)	A/A (n=46)	Diff (*p*)	G-car (n=144)	A/A (n=36)	Diff (*p*)	G-car (n=232)	A/A (n=46)	Diff (*p*)
Age, Y(SD)	86(6)	87(5)	0.12	89(6)	89(6)	0.73	91(6)	90(5)	0.45
Education, Y(SD)	17(4)	17(3)	0.91	16(4)	17(3)	0.58	16(3)	16(4)	0.68
Sex	122 F, 79 M	30 F, 16 M	0.62	90 F, 54 M	21 F, 15 M	0.70	159 F, 73 M	30 F, 16 M	0.73
APOE ε4, N(%)	33(17)	5(11)	0.50	33(23)	11(32)	0.39	81(35)	18(39)	0.62
MMSE (SD)	29(2)	28(2)	0.54	28(2)	28(2)	0.48	25(5)	26(4)	0.47
BMI (SD)	27(5)	27(5)	0.48	27(5)	26(5)	0.57	26(5)	25(4)	0.34
Systolic BP (SD)	134(19)	131(16)	0.20	137(18)	137(16)	0.90	137(18)	139(18)	0.58
Diastolic BP (SD)	71(11)	72(10)	0.63	70(14)	72(9)	0.33	71(12)	72(12)	0.69

Note: Continuous variables (age, education, BMI, MMSE, and BP) were analyzed for genotypic group differences using a student's t-test (two-tailed). Factor variables (sex and APOE ε4 status) were analyzed using Fisher's exact test (two-tailed). SD = standard deviation; car = carrier; CN = cognitively normal; MCI = mild cognitive impairment; AD = Alzheimer's disease; Y = years; M = male; F = female; MMSE = Mini Mental Status Exam; BMI = body mass index (height(cm)/weight(kg)^2); BP = blood pressure. All subjects were of Caucasian ancestry.

4.3.4 Statistical Analysis

4.3.4.1 Neuroimaging (CAMH and Zucker Hillside Samples)

Each sample was analyzed independently using the same approach; voxel-wise DTI analysis was carried out using TBSS (S. M. Smith et al., 2006), part of FMRIB's Software Library (FSL) (S. M. Smith et al., 2004). The outcome measure for DTI analysis was fractional anisotropy (FA), which measures the degree of directionality of water diffusion in the brain and is thought to be an indicator of microstructural tissue integrity (affected by fibre density, axonal diameter, and extent of myelination) (Beaulieu, 2002). FA images were created by fitting a tensor model to the raw diffusion data using FMRIB's Diffusion Toolbox (FDT), and then brain-extracted using the FSL Brain Extraction Tool (BET) (S. M. Smith, 2002). All subjects' FA data were then aligned

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to the FMRIB58 FA standard using FMRIB's Nonlinear Image Registration Tool (FNIRT), which uses a b-spline representation of the registration warp field. After affine registration to Montreal Neurological Institute 152 (MNI152) space, the mean FA image was created and thinned to form a mean FA skeleton representing the centers of all tracts common to the group (using a masking threshold of 0.2). Each subject's aligned FA data was then projected onto this skeleton and the resulting data were analyzed using voxel-wise cross-subject statistics (general linear models, co-varying for age, *APOE* ε4 status, and sex). For *post hoc* analysis, peak significant voxels within major white matter tracts were selected, and FA values at these voxels used as outcome measures in linear regression models to measure genotypic effects in relation to age.

Due to near-perfect linkage of the 5' *SORL1* haplotype (SNPs 8-10), all individuals were grouped according to rs689021 genotype using dominant (major allele [G] homozygotes vs. minor [A] carriers) and recessive (minor allele [A] homozygotes vs. major allele [G] carriers) models to determine the direction of effect. 5000 permutations were performed for each contrast and voxels were deemed significant if $p<0.05$ after threshold-free cluster enhancement (TFCE) correction for multiple comparisons across space. In both samples, *post hoc* analysis was performed for peak voxels within select tracts using OLS regression (R statistical software v.2.15.1) to visualize how genotype related to FA across age, using voxel FA as the dependent measure, co-varying for sex and *APOE* ε4 status.

4.3.4.2 Postmortem SORL1 mRNA (BrainCloud Sample)

The only *SORL1* SNP (within the SNP 8-10 haplotype) available in the Braincloud sample was rs689021 (SNP 9). Raw data were extracted and analyzed externally using R. Ordinary least squares (OLS) regression models were used, including restricted cubic splines to evaluate non-linear effects and interactions of genotype and age within ethnic subgroups (Caucasian and African American (AA)) together and separately, co-varying for sex, postmortem interval, and sample pH. Samples with an RNA integrity number (RIN) (Schroeder et al., 2006) of less than 7.0 were excluded from analysis to help reduce confounding due to poor RNA quality.

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4.3.4.3 Postmortem Amyloid Load and Tangles (Religious Orders Study {ROS} and Memory and Aging Project {MAP} Sample)

Of the total 710 subjects, 5 (0.7%) had non-conforming *SORL1* 5' haplotypes and were therefore excluded from analysis, resulting in a final n=705 for which SNPs 8-10 were in perfect LD. For neuropathology measures, the distributions of Aβ and PHFtau were heavily right skewed. We therefore performed median splits of each measure to create binary factors with values corresponding to zero-low and moderate-high pathology levels. The resulting data were analyzed using logistic regression to model these levels of Aβ and PHFtau as a function of *SORL1* rs689021 (SNP 9) genotype group, using an additive model with three genotypic groups, then using dominant (major allele [C] homozygotes vs. minor [T] carriers) and recessive (minor allele [T] homozygotes vs. major allele [C] carriers) models, co-varying for age, *APOE* ε4 status, sex, and education. Analysis was performed separately within each diagnostic group (CN, MCI, AD), and correction for multiple comparisons (2 pathological measures x 3 diagnostic groups=6 independent tests) was performed using FDR with q=0.05.

Following consistent evidence in existing literature of a strong and robust LD structure within the 5' region of *SORL1* (specifically the haplotype defined by SNPs 8-10) (Caglayan et al., 2012; Feulner et al., 2010; Kimura et al., 2009; Meng et al., 2007; Tan et al., 2009), as well as our own findings of near perfect LD within each analyzed sample (rare haplotype group frequencies were prohibitively low (<1%) and only present in the CAMH and ROS/MAP datasets), we chose to analyze one representative SNP across all four samples (SNP9, rs689021).

4.4 Results

4.4.1 Neuroimaging Samples (CAMH and Zucker Hillside Samples)

In both the CAMH and Zucker Hillside samples, the 5' haplotype block (SNPs 8-10) showed significant associations with white matter FA (Figure 4-1), with rs689021 A allele homozygotes showing reduced FA primarily in fronto-temporal white matter tracts, including the bilateral superior longitudinal fasciculus, uncinate fasciculus, inferior fronto-occipital fasciculus, and cingulum bundle, as well as right inferior longitudinal fasciculus, and the genu and splenium of the corpus callosum in both samples at 5% family-wise error (FWE) corrected thresholds.

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Additionally, the Zucker Hillside sample showed effects of genotype within the internal capsule. No effects of SNPs 23-25 were found in the CAMH sample. *Post hoc* analysis revealed a pattern of reduced FA in rs689021 A-allele homozygotes that was consistent across the age-range of both samples (i.e. no interaction with age) (Figure 4-2).

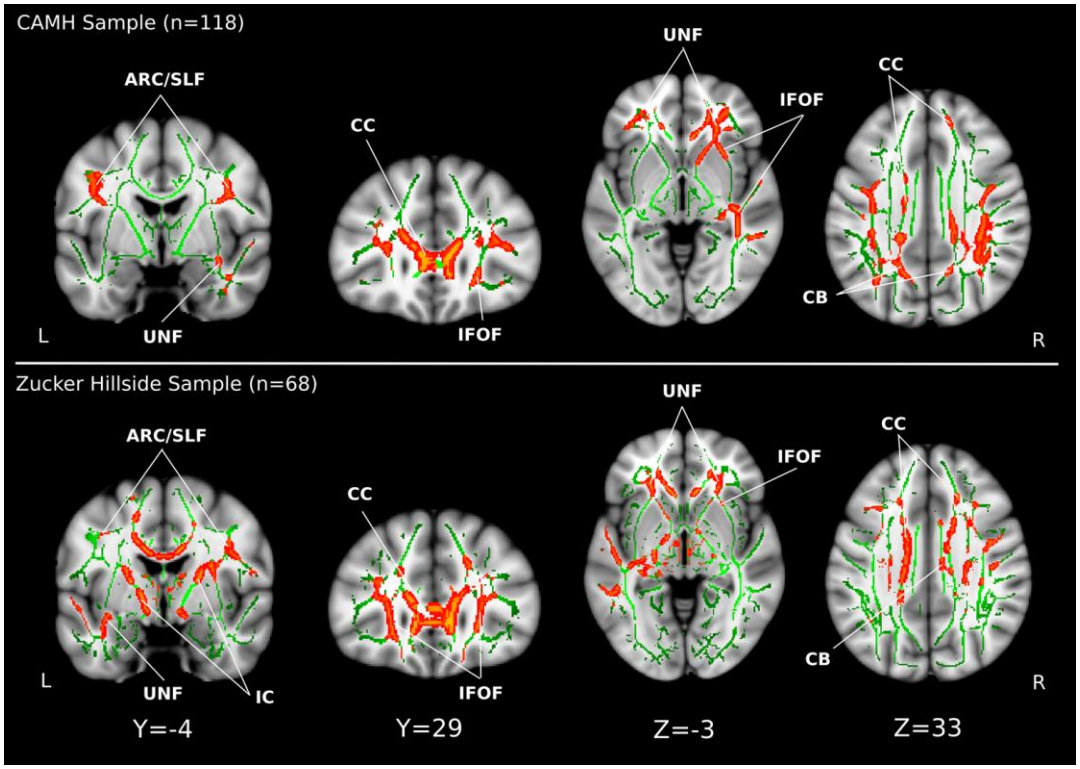

Figure 4-1. Results of TBSS white matter analysis for CAMH (A) and Zucker Hillside (B) imaging-genetics datasets. The average white matter FA skeletons for each sample have been overlaid on the MNI152 1mm T1-weighted brain standard and significant voxels are indicated by yellow-red colouring, corrected for multiple comparisons using TFCE at $p<0.05$. Only voxels within the mean FA skeleton (Green) were analyzed, surrounding voxels have been colo ured for emphasis. UNF = uncinate fasciculus; IFOF = inferior fronto-occipital fasciculus; CB = cingulum bundle; CC = corpus callosum; IC = internal capsule; ARC/SLF = arcuate fasciculus/superior longitudinal fasciculus; (R) = right; (L) = left.

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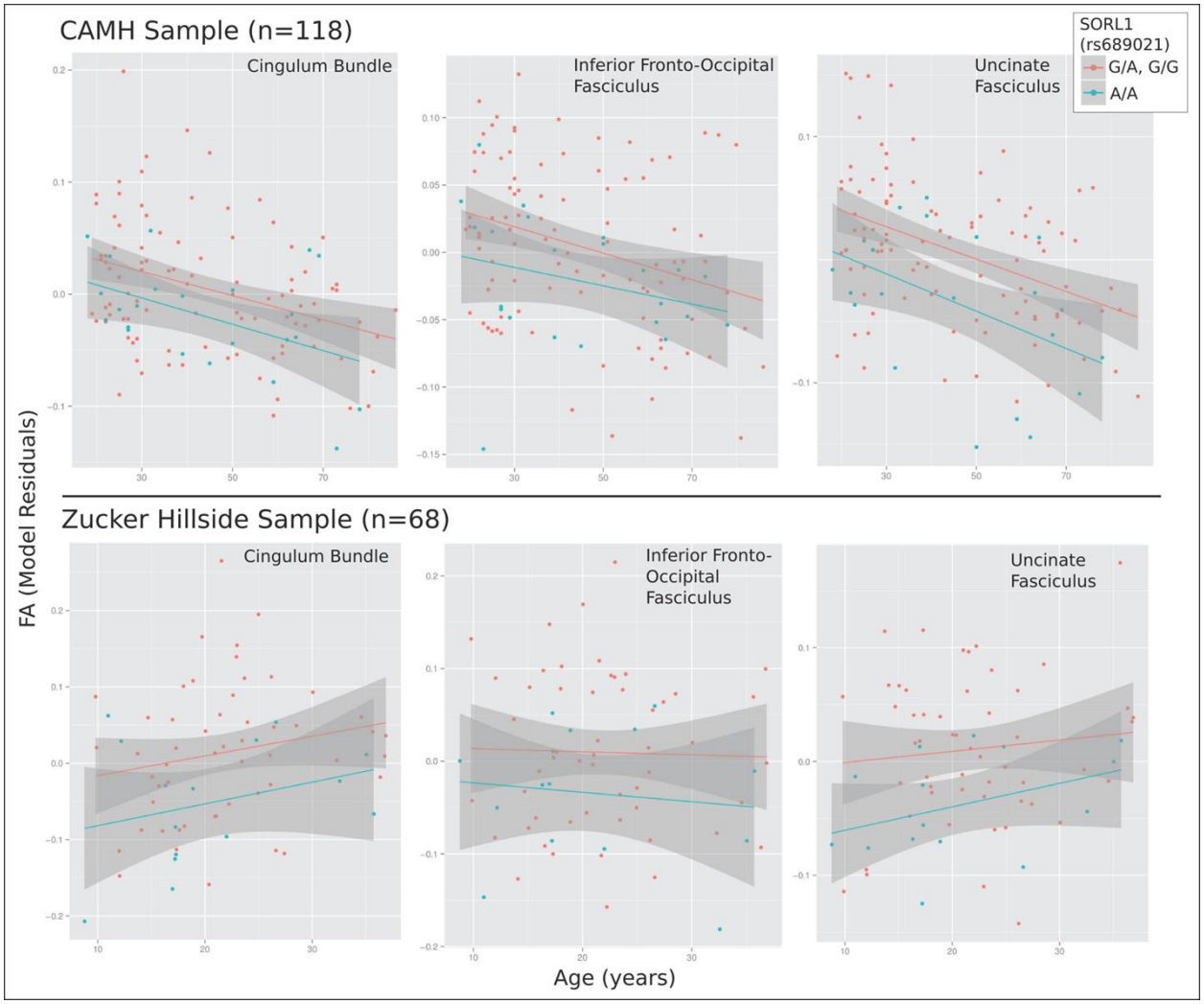

Figure 4-2. Regression model residuals of white matter fractional anisotropy at select peak voxels (as determined using TBSS) plotted against age, according to *SORL1* rs689021 genotypic group ([A] allele homozygotes vs. [G] allele-carriers) in both the CAMH and Zucker Hillside samples. Models co-varied for sex and *APOE* ε4 status.

4.4.2 Postmortem SORL1 mRNA (BrainCloud Sample)

After removing observations with RIN<7.0, age<0, and missing sample PH information, ethnic subgroup sample sizes were 3 (Asian), 5 (Hispanic), 90 (Caucasian), and 99 (AA). Based on these group sizes, analysis was conducted in the Caucasian and AA subgroups only. In the combined Caucasian and AA sample (n=189), a significant non-linear genotype by age interaction was found ($F_{12,176}$=4.06, p=0.008), co-varying for ethnicity, pH, PMI and sex, whereby major differences in *SORL1* mRNA levels were prominent during childhood and

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adolescence into early adulthood. During this period, the A-allele homozygotes demonstrated reduced prefrontal *SORL1* mRNA. Analyzing ethnic subgroups separately revealed that the effect was driven by Caucasians ($F_{11,78}$=7.03, p=0.0003) (Figure 4-3). No effect of *SORL1* variation was found in the AA group ($F_{11,87}$=0.1, p=0.97).

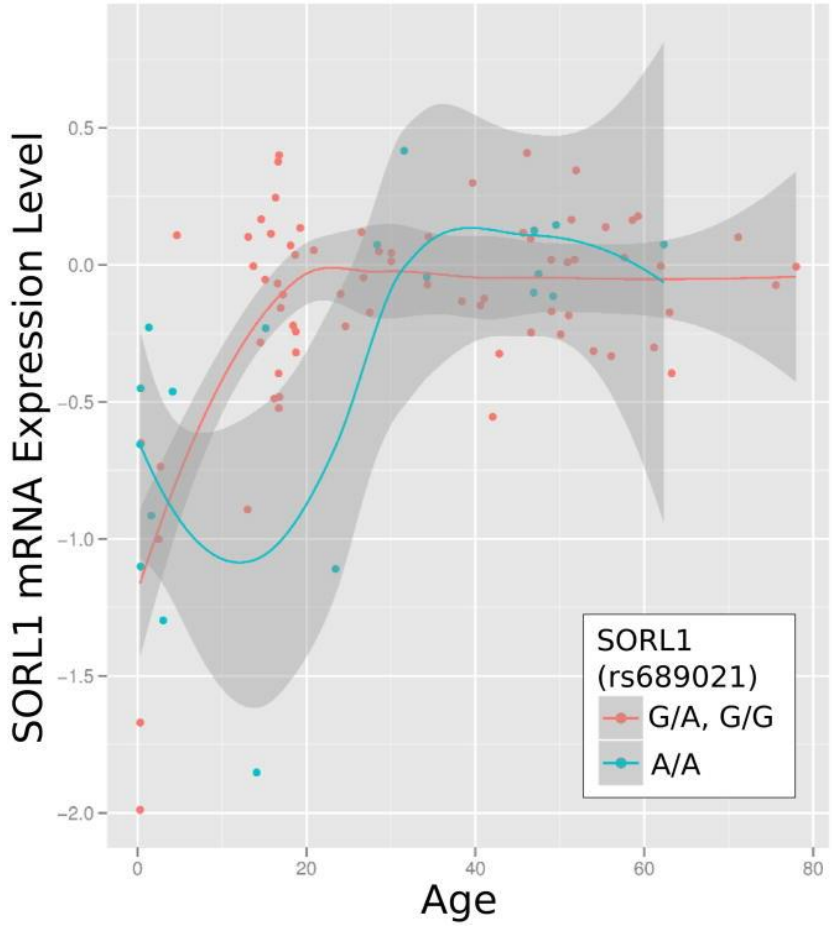

Figure 4-3. *SORL1* mRNA expression in the prefrontal cortex plotted against age, according to SNP 9 (rs689021) genotype in the BrainCloud postmortem sample. Raw expression data are shown fit with loess smoothing curves for each genotype. Ordinary least squares regression model shows a non-linear genotype by age interaction (interaction effect: $F_{11,78}$=7.03, two-tailed p=0.0003).

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4.4.3 Postmortem Amyloid Load and Tangles (Religious Orders Study {ROS} and Memory and Aging Project {MAP} Sample)

Associations of SORL1 rs689021 (SNP 9) genotype with Aβ were found in both MCI (dominant model, GG genotype<A carriers, O.R.=0.34 (95% C.I.=0.16-0.73), p=0.0056 (p_{adj}=0.03)) and AD (recessive model, AA genotype>G carriers, O.R. = 3.05 (95% C.I.=1.29-7.22), p=0.011 (p_{adj}=0.03)) subjects, but not in the CN group (genotypic model O.R.=1.2, p=0.65). For PHFtau**,** an association trend with rs689021 genotype was found in CN subjects (genotypic model, AA>GG, O.R.=2.26 (95% C.I.=0.98-5.21) , p=0.055 (p_{adj}=0.11)), but not in the MCI (genotypic model O.R.=0.96, p=0.93) or AD (genotypic model O.R.=1.41, p=0.42) groups. While the PHFtau result did not survive FDR correction, it is worth noting that the same T allele associated with greater Aβ pathology was also associated with increased PHFtau.

4.5 Discussion

We found that *SORL1* risk variants influenced microstructure of white matter tracts with known susceptibility in AD, in both imaging-genetics datasets, with consistent effect from childhood onward. We then bridged the gap from genetic risk variants to brain structure by demonstrating that the same *SORL1* risk variant predicted lower levels of mRNA expression across the lifespan, most prominently in childhood and adolescence, demonstrating a temporal consistency of onset of neural risk with our findings in both neuroimaging samples. Finally, we demonstrated that variation at the *SORL1* gene predicts amyloid-β plaque levels, thus conferring neuropathological risk via the amyloidogenic pathway.

In both the CAMH and Zucker Hillside samples, *SORL1* risk variants were associated with lower white matter FA in structures vulnerable in MCI and the earliest phases of AD. Conventional MRI studies show brain changes in AD typically occur first in medial temporal structures, spreading globally as the disease progresses (de Leon et al., 2004; Clifford R. Jack et al., 2004; Karas et al., 2004). DTI studies in AD have shown that this gray matter neurodegeneration is paralleled by impairment in white matter tract microstructure (i.e. FA), primarily in association fibers connecting to the medial and lateral temporal lobes (H. Huang et al., 2012). These changes are also present in MCI individuals who have not yet developed dementia (Pievani et al., 2010;

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Y. Zhang et al., 2009). The results of a recent study, which identified parahippocampal white matter FA (part of the cingulum bundle in the medial temporal lobe), as the single best neuroimaging predictor of incipient cognitive impairment (Carmichael & Salloway, 2012) raise the possibility that white matter changes may precede gray matter changes in the sequence of preclinical AD-related neural events. Our data support that the very earliest forms of genetically-mediated neural risk for AD may occur through white matter pathways, from childhood onward. A previous examination of *SORL1* and white matter found increased risk for postmortem white matter atrophy and white matter hyperintensities in elderly individuals *in vivo* in the elderly white MIRAGE (Multi-Institutional Research in Alzheimer's Genetic Epidemiology) cohort (Cuenco et al., 2008). Although white matter hyperintensities can be present earlier in adult life, they are generally uncommon in healthy young individuals (Hopkins et al., 2006), and as such, may not be as useful as microstructural integrity of white matter when assessing subtle forms of early neural risk for AD.

Our lifespan analysis using BrainCloud demonstrates that the effects of *SORL1* risk variants on *SORL1* mRNA expression are most prominent from childhood through to early adulthood (i.e. during neurodevelopmental phases of the lifespan). Minor allele homozygotes showed reduced mRNA expression during this period in the lifespan, consistent with our findings of reduced microstructural integrity of white matter already present from childhood onward in the Zucker Hillside sample and from late adolescence onward in the CAMH sample. Previous studies have found allelic differences in SORL1 protein (Caglayan et al., 2012) and mRNA levels (McCarthy et al., 2012) in elderly postmortem brain; however, by using a lifespan approach, we provide the first evidence that the temporal impact of *SORL1* risk variants on *SORL1* mRNA expression occurs during neurodevelopmental phases of the lifespan, rather than in late-life.

Our association of *SORL1* genotype with amyloid-β plaque levels provides direct neuropathological evidence that *SORL1* confers risk for AD through the amyloidogenic pathway. Our results confirm those of *in vitro* studies which have found that increased levels of SORL1 result in decreased APP processing (V. Schmidt et al., 2012) and greater production of intracellular $A\beta_{42}$ (Ma et al., 2009; V. Schmidt et al., 2012). Loss of SORL1 expression in histologically normal late-onset AD brain-derived neurons (Dodson et al., 2006; Scherzer et al., 2004) suggests that this is a primary event in late-onset AD pathology and may precede disease onset. SORL1's role in amyloid accumulation supports its role as a risk factor for AD rather than

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as a marker of disease progression. Our findings do not support SORL1 as a marker of disease progression (i.e. accumulation of tau pathology) in AD populations, which have recently been shown to be due to an entirely different set of genetic factors (Cruchaga et al., 2010). Although it is possible that subtle changes in $A\beta_{42}$ concentration resulting from allelic differences in SORL1 expression drive changes in microstructural integrity of white matter early in life, our study cannot directly answer this question. Indirect evidence for this possibility is provided by inverse correlations of CSF levels of SORL1 protein with $A\beta_{42}$ in MCI subjects (Alexopoulos et al., 2012), and association of CSF levels of $A\beta_{42}$ with medial frontal FA (Bendlin et al., 2012).

There are several potential limitations to this study. First, in healthy control samples, it is possible that subclinical symptomatology might be present, and this caveat should be taken into consideration when interpreting our results. However, the similar results in both of our neuroimaging samples, which were from different countries and of different age range, provide added confidence in our results. Second, as with any group-wise analysis of means, the relatively small group sizes of risk allele homozygotes in some of our samples can be considered a limitation. However, statistically significant associations were found in each sample, and the direction of effect was consistent across samples. Third, due to the cross-sectional nature of our analyses, we cannot unequivocally conclude that the imaging results are specific to risk for AD, as white matter impairments are prevalent in other disorders, such as depression, that are known to affect older adults (White, Nelson, & Lim, 2008). It is important to note, however, that *SORL1* is considered an Alzheimer's risk gene, based both on genome-wide analysis and meta-analysis (Reitz et al., 2011). Furthermore our findings align with previous investigations of regions/tracts that are first affected in early AD and MCI, such as the cingulum bundle, (Carmichael & Salloway, 2012; Y. Zhang et al., 2007) uncinate fasciculus,(Larroza, Moratal, D'ocón Alcañiz, Arana, & por la Alzheimer's Disease Neuroimaging Initiative, 2013; Morikawa et al., 2010) and corpus callosum (J.-H. Wang et al., 2013).

Importantly, our findings must be viewed in context of the existing literature. In the initial Rogaeva et al. (Rogaeva et al., 2007) study (as well as the Reitz et al. meta-analysis (Reitz et al., 2011)), the SNP 8-10 haplotype associated with increased risk for AD diagnosis was CGC. In the Cuenco et al. (Cuenco et al., 2008) imaging study, it is the A allele at SNP 9 (corresponding to the TAT haplotype) that is associated with increased risk for AD-associated imaging phenotypes (notably white matter atrophy and hyperintensities), and the T allele at SNP 8 (belonging to the

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same TAT haplotype) was associated with smaller hippocampal volumes in the only other imaging investigation of *SORL1* gene variants by Bralten et al. (Bralten et al., 2011). Our neuroimaging results, along with our results of mRNA expression and beta-amyloid, are in agreement with these existing structural imaging findings within the 5' region of *SORL1*. Therefore, when all genetic investigations of *SORL1* are taken together, it appears that allelic heterogeneity may be operating at these loci.

The demonstrated effects of *SORL1* variation on brain structure, *SORL1* mRNA, and amyloid pathology coupled with our lifespan approach, provide answers about when, where, and how this gene confers neural risk for AD. Our study identifies *SORL1*-related risk mechanisms and neuroimaging biomarkers that can be utilized in potential intervention studies targeted toward risk carriers, yet our findings also raise questions regarding when in the lifespan such interventions should be tested. At the same time, it is clear that variation at the *SORL1* gene, except for rare cases of identified mutations, is unlikely to act as a causative factor alone for late-onset AD. Therefore, systematic assessment of other risk genes using similar multi-level lifespan approaches are first required to move closer toward targeted genetically-based interventions in healthy individuals at-risk for late-onset AD.

4.6 Acknowledgements

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Within the past five years, BGP has been a member of the advisory board of Lundbeck Canada (final meeting was May 2009) and Forest Laboratories (final meeting was March 2008). He has also served one time as a consultant for Wyeth (October 2008) and Takeda (July 2007), and was a faculty member of the Lundbeck International Neuroscience Foundation (LINF) (final meeting was April 2010). JLK has been a consultant to GlaxoSmithKline, Sanofi-Aventis, and Dianippon-Sumitomo. BHM has received travel support from Roche. AKM has served as a consultant for Genomind Inc. ANV had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Chapter 5

5 Cerebrovascular and Microglial States are Not Altered by Functional Neuroinflammatory Gene Variant

The contents of this chapter have been accepted for publication in the Journal of Cerebral Blood Flow and Metabolism as a Negative Report:

Felsky D et al. Cerebrovascular and Microglial States are Not Altered by Functional Neuroinflammatory Gene Variant.

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5.1 Abstract

Alzheimer's disease is characterized by the accumulation of plaque and tangle neuropathology as well as chronic neuroinflammation. The translocator protein (TSPO) is thought to be a marker for neuroinflammation; recent studies have implicated TSPO in multiple neurological and psychiatric conditions with immunological components (e.g. Bipolar disorder, Alzheimer's disease). Known links between vascular factors, inflammation, and dementia have solidified cerebrovascular disease phenotypes (in particular white matter hyperintensities and cerebral infarcts) as markers of Alzheimer's disease risk and progression. The *TSPO* rs6971 polymorphism reliably determines binding of multiple TSPO radioligands in the brain, however, it is not known if this change in binding affinity affects pathological or inflammatory processes important in neurodegenerative disease and no study to date has examined the relationship between rs6971 and structural neuroimaging, postmortem neuropathology, or inflammatory biomarkers. We performed comprehensive analyses of the effects of rs6971 on *in vivo* white matter hyperintensities, as well as both *in vivo* and postmortem cerebral infarcts. To examine potential mechanisms, we also analyzed the effects of rs6971 on plasma inflammatory biomarkers in living subjects and microglial density at three stages of activation measured from postmortem brain tissue. In two large, independent elderly cohorts (ADNI 1, GO, and 2: n_{total}=1 330; ROS/MAP: n_{total}=1 015), we found no significant associations of genotype with cerebral infarcts (either postmortem or *in vivo*) or white matter hyperintensities. Further, we found no replicated genotype effects on plasma inflammatory biomarkers, cerebral amyloid angiopathy, or microglial activation. Taken together, our results do not support a contribution of rs6971 to cerebrovascular and inflammatory risk factors implicated in Alzheimer's disease.

5.2 Introduction

Late-onset Alzheimer's disease is characterized by the accumulation of beta-amyloid plaques and neurofibrillary tangles, chronic inflammation, and progressive neurodegeneration (Akiyama, 1994; Heneka, O'Banion, Terwel, & Kummer, 2010; Hommet et al., 2014; Rubio-Perez & Morillas-Ruiz, 2012). The inflammation hypothesis of Alzheimer's disease posits that chronic over-activation of microglia in response to the buildup of amyloid pathology results in a positive

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feedback loop whereby pro-inflammatory conditions sustain the elevated expression of factors that further exacerbate amyloidogenesis (Akiyama, 1994; Bagasra et al., 1995; Kummer et al., 2011, 2012). Accordingly, serum levels of high-sensitivity C-reactive protein have been shown to increase risk for Alzheimer's diseaseup to three-fold (R. Schmidt et al., 2002), and some evidence suggests that non-steroidal anti-inflammatory drugs may both reduce the likelihood of developing Alzheimer's disease if taken by pre-symptomatic healthy adults (Breitner et al., 2011; Vlad, Miller, Kowall, & Felson, 2008) and slow the rate of cognitive decline in patients (Rich et al., 1995) (potentially through both amyloidogenic (Sastre et al., 2006) and inflammatory processes (McGeer & McGeer, 2007; Vlad et al., 2008)). In addition to the damaging effects of "traditional" neuroinflammation, the immune response also serves to protect against neurodegeneration; studies show that increased expression of certain inflammatory cytokines may reduce Aβ deposition (Chakrabarty, Herring, Ceballos-Diaz, Das, & Golde, 2011) and play roles in promoting cell survival and neurovascularization (Glass, Saijo, Winner, Marchetto, & Gage, 2010; Rubio-Perez & Morillas-Ruiz, 2012).

The convergence of inflammation and cerebrovascular pathology in Alzheimer's disease is becoming increasingly clear, yet the mechanisms behind this convergence are poorly understood. It is known that cerebral ischemia is a strong activator of the immune response (del Zoppo et al., 2000; Iadecola & Alexander, 2001), and that, in parallel, vascular irregularities (e.g. hypertension/hypotension, hypercholesterolemia, ischemic stroke, transient ischemic attack, diabetes, cardiac disease) are themselves risk factors for Alzheimer's disease (Casserly & Topol, 2004; de la Torre, 2002). Amyloid deposition within neurovasculature, known as cerebral amyloid angiopathy (CAA), may contribute to brain ischemia and lead to cognitive impairment (Greenberg, Gurol, Rosand, & Smith, 2004; Viswanathan & Greenberg, 2011). Cerebral infarcts are regions of ischemic damage associated with aging and present to a greater degree in individuals who develop Alzheimer's disease than in those who do not (Vermeer et al., 2003). The presence of both macro and micro cerebral infarcts, caused largely by cerebral small vessel disease (SVD) (Pantoni, 2010), have been shown to influence risk for Alzheimer's disease as well as global cognitive performance (Arvanitakis, Leurgans, Barnes, et al., 2011; J. A. Schneider et al., 2003; van Rooden et al., 2014), and may be a consequence of accumulating cerebrovascular insults (and accompanying hypoperfusion (Suter et al., 2002)) that are implicated in neurodegenerative processes (Farkas & Luiten, 2001). At the neuroimaging level,

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white matter lesions associated with SVD can be imaged *in vivo* as regions of increased signal on T2-weighted MR images; these white matter hyperintensities (WMH) have been associated with increased age, the presence of cerebral infarcts, multiple vascular disease risk factors, Alzheimer's disease and mild cognitive impairment (MCI) diagnosis, and the progression from MCI to Alzheimer's disease (Brickman, 2013; Yoshita et al., 2006).

At the molecular genetic level, the translocator protein 18kDa (TSPO, formerly known as the peripheral benzodiazepine receptor (PBR)) has recently become a focus of interest in the investigation of psychiatric and neurological illnesses with potential immune mechanisms of illness. TSPO is expressed in activated microglia and used as a biomarker for neuroinflammation (Cagnin et al., 2001; Venneti et al., 2006); it is found in the outer mitochondrial membrane where it is thought to regulate cholesterol transport, steroidogenesis, and apoptosis (Veenman, Papadopoulos, & Gavish, 2007). *In vivo* positron emission tomography (PET) imaging studies of TSPO radioligands show increased binding in patients with acute brain injury (M.-K. Chen & Guilarte, 2008), multiple sclerosis (Banati et al., 2000; Harberts et al., 2013; Oh et al., 2011), mild cognitive impairment (MCI) (Yasuno et al., 2012) and Alzheimer's disease (Cagnin et al., 2001; Kreisl et al., 2013). TSPO binding is increased in Alzheimer's disease subjects compared to controls and is correlated with disease severity (Kreisl et al., 2013). Consistent with TSPO's proposed role in neuroinflammation, TSPO binding is increased specifically in white matter in patients with multiple sclerosis (Colasanti et al., 2014; Takano et al., 2013). A single genetic polymorphism located in exon 4 of the *TSPO* gene, rs6971 (Ala147Thr), reliably determines the binding affinity of second generation TSPO radioligands in the brain (Mizrahi et al., 2012; Owen et al., 2012), where A/A, A/G, and G/G genotypes correspond to high, medium, and low affinity binding phenotypes (herein referred to as HABs, MABs, and LABs, respectively). It is hypothesized that the Alanine to Threonine substitution at position 147 results in a conformational change in TSPO structure that influences its interaction with a variety of molecules (Korkhov et al., 2010; Murail et al., 2008; Owen et al., 2012). This difference in ligand affinity may have important implications for the etiopathology of Alzheimer's disease; TSPO ligands have been shown to ameliorate neuroinflammation *in vitro* (Karlstetter et al., 2014), reverse neuropathology and behavioral decline in Alzheimer's disease mouse models (Barron et al., 2013), reduce gamma radiation-induced apoptosis, Aβ42-induced neurodegeneration, and premature death in drosophila (R. Lin et al., 2014), as well as confer

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neuroprotective and regenerative effects *in vivo* and *in vitro* (Ferzaz et al., 2002; Girard et al., 2008; Ryu et al., 2005; Veiga et al., 2005).

Despite this potential involvement of TSPO in the etiopathology of neurodegenerative disorders, and the well-known links between inflammation, cerebrovascular disease, and AD, no studies to date have investigated the effect of rs6971 on MRI-based phenotypes or inflammatory biomarkers. We therefore sought to test whether *TSPO* rs6971 genotype was associated with *in vivo* structural imaging measures of cerebrovascular disease and neuroinflammation in two large clinical samples (the Alzheimer's Disease Neuroimaging Initiative (ADNI) and the Religious Orders Study / Memory and Aging Project (ROS/MAP)). To identify potential mechanisms of action, we also analyzed the effect of genotype on levels of plasma inflammatory biomarkers in living subjects, as well as on cerebral infarcts, CAA, and densities of active microglia from postmortem brain tissue. Due to the anti-inflammatory action of TSPO ligands (Leaver et al., 2011), we hypothesize that that low-affinity binding groups would have exacerbated pathology and increased levels of pro-inflammatory biomarkers vs. medium- and high-affinity binding groups, as determined by genotype.

5.3 Methods

5.3.1 Alzheimer's Disease Neuroimaging Initiative (ADNI)

Subject Characteristics: The Alzheimer's Disease Neuroimaging Initiative (ADNI) is a multi-center collaboration established in 2003, in which elderly subjects at various stages of cognitive impairment are assessed longitudinally for multi-modal imaging, neuropsychiatric test performance, and fluid biomarkers. Clinical evaluations were administered to each subject at enrollment by trained physicians as described (Petersen et al., 2010). A total of 699 individuals (195 cognitively normal (CN), 338 late mild cognitive impairment (LMCI), and 166 Alzheimer's disease subjects) from ADNI 1 and 631 individuals from ADNI GO and 2 (129 CN, 40 significant memory concern (SMC), 242 early mild cognitive impairment (EMCI), 122 LMCI, and 98 Alzheimer's disease subjects), all self-reported Caucasian, with baseline data for white matter hyperintensity volume (WMHv) and genetics were included in analyses (ntotal=1 330). A subset of subjects from ADNI 1, GO, and 2 also had data for cerebral infarcts (ntotal=1 151).

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Data were extracted from the ADNI website (http://adni.loni.usc.edu) in the form of the ADNIMERGE 0.0.1 package for R. Details on subject recruitment and inclusion/exclusion criteria are reported elsewhere (Petersen et al., 2010).

Genetics: All subjects were genotyped using the Human610-Quad BeadChip assay (Illumina, Inc., San Diego, CA), which included the rs6971 SNP. *APOE* ε4 status was obtained separately by genotyping rs429358 and rs7412 using PCR and HhaI restriction enzyme digestion, according to previously published methods (Saykin et al., 2010).

5.3.2 Religious Orders Study / Memory and Aging Project (ROS/MAP)

Subject Characteristics: The Religious Orders Study (ROS) and Memory and Aging Project (MAP) are community-based cohort studies of aging and dementia. Participants in ROS are older nuns, priests and brothers from across the US (Bennett, Schneider, Arvanitakis, et al., 2012), and those in MAP are older residents of northeastern Illinois (Bennett, Schneider, Buchman, et al., 2012). Both studies were approved by the Institutional Review Board of Rush University Medical Center and enroll older persons without dementia who agree to annual evaluation and autopsy. The follow-up rate exceeds 90% and the autopsy rate exceeds 85%. A neuroimaging sub-study was initiated in 2009 (Fleischman et al., 2014).

A total of 1 015 postmortem brains were available for analysis at time of these analyses. All subjects were assessed with a comprehensive uniform, structured, clinical evaluation that included a self-report medical history obtained by trained nurses and research technicians, a neurologic examination by trained nurses and cognitive function testing by trained neuropsychological test technicians. Full details on sample characterization and assessments have been previously published (Bennett et al., 2006).

Genetics: All subjects were genotyped using the Affymetrix (Santa Clara, CA, USA) Genechip 6.0 platform. *TSPO* rs6971 was directly genotyped. *APOE* (rs7412 and rs429358) genotypes were imputed from MACH (version 1.0.16a) and HapMap release 22 CEU (build 36), as previously described (Chibnik et al., 2011).

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5.3.3 *In Vivo* Neuroimaging

5.3.3.1 ADNI

Neuroimaging acquisition: Structural MRI images (including T1, T2, PD) were acquired for all subjects using a standard protocol on 1.5T scanners across multiple sites. Correction for gradient non-linearity was performed using gradwarp and standardization across sites and platforms was performed as previously published (Clifford R. Jack et al., 2008).

Cerebral infarct quantification: The presence of cerebral infarcts was evaluated for all subjects in ADNI 1, GO, and 2 using the same method, outlined by De Carli et al. on the ADNI website (DeCarli, Carmichael, & He, 2013). Each subject's MR image set was evaluated by a specially trained physician, and the presence of MRI infarction was determined based on the size, location, and imaging characteristic of the lesion. Only lesions 3mm or larger qualified for consideration as cerebral infarcts. Inter-rater reliability for detection of infarcts as assessed with the kappa statistic was generally high (between 0.73 and 0.90), consistent with previous studies (DeCarli et al., 2005; Hachinski et al., 2006).

WMH volume estimation: For ADNI 1 – WMHv estimates were derived from T1, T2, and PD images using a method described elsewhere (Schwarz, Fletcher, DeCarli, & Carmichael, 2009). This method uses models of WMH spatial distributions from a training dataset of "ground truth" FLAIR-based WMH detections (derived from a strongly-validated semi-automated protocol (Yoshita, Fletcher, & DeCarli, 2005)). These models are combined with a probabilistic model of the PD, T1, and T2 intensity distributions in a Bayesian Markov Random Field framework that allows for inference of WMH positions in novel subject images. The output standardized WMHv data used for ADNI1 analyses were available in the "ucd_adni1_wmh" file downloaded as part of the ADNIMERGE (0.0.1) R package.

For ADNI GO and 2 - Volumes for CSF, gray matter, white matter, and WMH were calculated from FLAIR and T1-weighted images using a four-tissue Bayesian segmentation method, outlined by De Carli et al. on the ADNI website (DeCarli, Maillard, & Fletcher, 2013). Briefly, FLAIR images were first affine transformed to T1-weighted images and inhomogeneity correction was performed (DeCarli et al., 1996). Images were then non-linearly registered to a standard template space and WMHv was estimated using a modified Bayesian probability

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structure based on a previously published method of histogram fitting (DeCarli et al., 1999). WMHv and other tissue segmentations were then reverse transformed to native space. Output unstandardized WMHv data used for ADNI GO and 2 analyses were available in the "ucd_adni2_wmh" file downloaded as part of the ADNIMERGE (0.0.1) R package.

5.3.3.2 ROS/MAP

Neuroimaging acquisition: A subset of subjects with genetic data (n=291) underwent a multi-modal neuroimaging protocol that included high-resolution T1-weighted magnetization-prepared rapid acquisition gradient-echo (MPRAGE) and T2-weighted fluid attenuated inversion recovery (FLAIR) scans. Detailed scan acquisition parameters have been published elsewhere (Arfanakis et al., 2013).

WMH volume estimation: WMHv estimates were extracted from scans using an automated pipeline, as described (Arfanakis et al., 2013). First, T1-weighted MPRAGE images were spatially registered to the T2-weighted FLAIR images using affine registration (FLIRT, FMRIB, University of Oxford, UK) (Jenkinson, Beckmann, Behrens, Woolrich, & Smith, 2012; S. M. Smith et al., 2004). Brain was then extracted using FSL's brain extraction tool (BET) (S. M. Smith, 2002) and WMHs were automatically segmented for each participant using a support vector machine classifier considering both T1-weighted MPRAGE and T2-weighted FLAIR information (WMLS, SBIA, University of Pennsylvania, PA) (Zacharaki, Kanterakis, Bryan, & Davatzikos, 2008).

5.3.3.3 Statistical Analysis

All statistical analyses were performed using R statistical software (version 3.0.2). The presence or absence of cerebral infarcts across ADNI 1, GO, and 2 was evaluated as a dichotomous outcome using logistic regression, with TSPO genotype (additive model), sex, age, diastolic blood pressure (hypertension y/n in ROS/MAP), education (total years), and *APOE* ε4 status as predictors. Post-hoc tests were performed to evaluate the effect of genotype within each diagnostic group separately.

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Ordinary least squares (OLS) regression was performed on baseline data to model the effect of *TSPO* genotype on WMHv. Due to important differences between white matter hyperintensity volume estimate methodologies between ADNI 1, ADNI GO and 2, and ROS/MAP, the datasets were analyzed separately. In all three datasets, the distributions of WMHv were right-skewed; therefore Box-Cox power transformations were applied (Box & Cox, 1964). The effect of *TSPO* genotype was evaluated using an additive model in all subjects (controlling for age, sex, diastolic blood pressure (hypertension y/n in ROS/MAP), education (years), and *APOE* ε4 status). Since the ADNI 2 FLAIR-derived WMHv estimates were not normalized to a standard space, models of this data co-varied for total intracranial volume. Post-hoc tests were carried out within each diagnostic group separately. Due to their biologically related functions in cholesterol transport (Dimitrova-Shumkovska, Veenman, Roim, & Gavish, 2013) and TSPO's ability to up-regulate APOE (Taylor et al., 2014), interactions between *APOE* ε4 status and *TSPO* genotype were also tested in each model.

5.3.4 *In Vivo* Plasma Biomarkers

5.3.4.1 ADNI 1

Data were available from a subset of individuals from ADNI 1 (n=520) who contributed plasma aliquots for proteomic analysis. The 190 analyte multiplex immunoassay panel, referred to as the human discovery map, was developed on the Luminex xMAP platform by Rules-Based Medicine (Myriad RBM, Austin, TX) to contain proteins previously reported in the literature as altered in cancer, cardiovascular disease, metabolic disorders and inflammatory conditions. The method uses a flow-based laser apparatus to detect fluorescent polystyrene microspheres which are loaded with different ratios of two spectrally distinct fluorochromes. Full protocol details are available through the ADNI website (http://adni.loni.usc.edu/wp-content/uploads/2010/11/BC_Plasma_Proteomics_Data_Primer.pdf). Concentrations of five inflammatory biomarkers were available that were also assayed in the ROS/MAP sample: tumor necrosis factor alpha (TNFα), interleukin-6 receptor (IL6R), C-reactive protein (CRP), vascular cell adhesion protein 1 (VCAM1, CD106), and matrix metallopeptidase 9 (MMP-9).

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5.3.4.2 ROS/MAP

A subset of subjects (n=394) had blood drawn and plasma inflammatory biomarkers quantified according to previously published methods (Arfanakis et al., 2013). Briefly, blood samples were collected using a standard protocol, then highly sensitive multiplexed sandwich ELISA arrays (Endogen Searchlight technologies, Billerica, MA) were used to detect plasma concentrations of five inflammatory proteins that were also assessed in the ADNI 1 plasma proteomic assay, as described above.

5.3.4.3 Statistical Analysis

OLS regression was used to analyze the additive effect of *TSPO* genotype on plasma concentrations of each biomarker within ADNI and ROS/MAP subsamples separately. Outcomes were tested for normality using the Shapiro-Wilk test (Royston, 1995) and corrected, if necessary (Shapiro-Wilk $p<0.01$), using Box-Cox power transformations (Box & Cox, 1964). P-values were corrected for five independent comparisons (five plasma proteins) using the Bonferroni method. Models co-varied for age, sex, and *APOE* ε4 status. Post-hoc tests were carried out within each diagnostic group separately and interactions of rs6971 genotype with *APOE* ε4 status were also considered.

5.3.5 Postmortem Neuropathology (cerebral infarcts, amyloid angiopathy, and microglial density), ROS/MAP

5.3.5.1 Neuropathological Evaluation

Brain autopsies were conducted at predetermined sites across the United States and neuropathological examinations were performed at Rush University Medical Centre. Full details of autopsy and pathological evaluation procedures have been previously published (Arvanitakis, Leurgans, Barnes, et al., 2011; Bennett et al., 2005).

Macro cerebral infarcts: brains were removed, cut coronally into 1cm slabs and examined by a certified neuropathologist: age, size, and location of all cerebral infarcts were documented. Slabs

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from one hemisphere were fixed for three days (4% paraformaldehyde) and were then further dissected. Suspected infarcts were excised, embedded in paraffin, and confirmed using hematoxylin & eosin stain.

Micro cerebral infarcts: a minimum of nine regions in a single hemisphere were examined on 6µm paraffin-embedded sections stained with hematoxylin/eosin. Age and location of micro infarcts were recorded. Because acute and subacute infarcts were unlikely to be related to dementia, we only considered chronic macro and micro infarcts; these included cavitated or incomplete infarcts, with few remaining macrophages and fibrillary gliosis. Both macro and micro cerebral infarcts were coded as either present (one or more infarcts) or absent, as previously reported (J. A. Schneider et al., 2003).

Cerebral amyloid angiopathy (CAA): For a subset of 894 subjects, tissue from five regions (midfrontal, inferior temporal, angular gyrus, and calcarine cortices, as well as hippocampus) was dissected from paraformaldehyde-fixed slabs, paraffin-embedded, cut into 20-µm sections, and mounted on glass slides. CAA was assessed in each region using immunohistochemical labeling with anti-Aβ (Clone 6F/3D, M 0872; DAKO; 1:100). Because CAA has been shown to be highly correlated across regions and most subjects showed some degree of pathology in this sample (Arvanitakis, Leurgans, Wang, et al., 2011), CAA was averaged across all five dissected regions and categorized into a 3-level factor variable, corresponding to 1) no-to-minimal, 2) mild-to-moderate, and 3) moderate-to-very severe pathology. Full details have been previously described (Arvanitakis, Leurgans, Wang, et al., 2011).

Quantification of activated microglia: Immunohistochemistry was performed on a subset of brains using an Automated Leica Bond immunostainer (Leica Microsystems Inc., Bannockborn IL) and anti-human HLA-DP, DQ, DR antibodies (clone CR3/43; DakoCytomation, Carpinteria CA; 1:100), according to methods described previously (Bradshaw et al., 2013). Briefly, the densities of microglia present at three stages of activation were quantified in four brain regions: inferior temporal, mid-frontal, posterior putamen, and ventral medial caudate. Different stages of microglial activation - from least (stage 1) to most (stage 3) activated - were defined based on morphological characteristics; when these cells become activated, their long fine processes contract and thicken and the cell body adopts a larger more rounded cellular conformation. Cell counts were made by a trained investigator (blinded to clinical and pathologic data) from two

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adjacent blocks of tissue within each region (0.5 to 1.0 cm apart), and were averaged to obtain composite average densities of microglia in each region.

5.3.5.2 Statistical Analysis

Logistic regression was used to evaluate the association of *TSPO* rs6971 genotype with the presence or absence of micro and macro cerebral infarcts. Genotype effects were initially tested using an additive model across all diagnostic groups, controlling for age at death, sex, hypertension (y/n), education (years), and *APOE* ε4 status. P-values were corrected for seven independent comparisons (brain-wide micro infarcts, macro infarcts, amyloid angiopathy, and microglial activation in four distinct regions) using the Bonferroni method. Post-hoc tests were carried out within each diagnostic subgroup separately. Ordinal regression was used to evaluate the degree of amyloid angiopathy across diagnostic groups using the same modeling approach and set of co-variates as above (for cerebral infarcts). Linear regression was used to evaluate the effect of genotype on total microglial density (all stages of activation), as well as the square root-transformed ratio of most active (stage 3) to least active microglia (stage 1), in each region, co-varying for age at death, sex, and *APOE* ε4 status. Interactions between *APOE* ε4 status and *TSPO* genotype were also considered for each model. For all analyses, P-values are two-tailed and reported as corrected (p_{cor}, using the Bonferroni method as specified in Methods) or uncorrected (p_{raw}).

5.4 Results

5.4.1 *In Vivo* Cerebral Infarcts and White Matter Hyperintensities (ADNI and ROS/MAP)

Table 5-1 summarizes demographic characteristics of the ADNI 1 and ADNI GO and 2 cohorts analyzed, according to *TSPO* rs6971 genotype.

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Table 5-1. Summary Statistics for ADNI Samples by Diagnosis

ADNI 1 (n=699)	CN (n=195)	SMC	EMCI	LMCI (n=338)	AD (n=166)	Diff *p*
WMHv* (SD)	0.8 (2)	-	-	0.9 (3)	1.3 (3)	0.07
CI (-/+)[a]	18+,177-	-	-	30+,308-	13+,153-	0.9
Age, Y (SD)	75 (7)	-	-	75 (7)	76 (7)	0.22
Sex (F/M)	88 F, 107 M	-	-	117 F, 221 M	75 F, 91 M	0.018
BP (systolic)	133 (15)	-	-	133 (17)	135 (18)	0.35
BP (diastolic)	73 (10)	-	-	74 (10)	73 (9)	0.61
MMSE (SD)	29 (1)	-	-	27 (2)	23 (2)	<0.0001
Education, Y (SD)	16 (3)	-	-	16 (3)	15 (3)	0.0002
***APOE* ε4 status (-/+)**	53+,142-	-	-	185+,153-	112+,54-	<0.0001
rs6971 genotype (LAB/MAB/HAB)	20/88/87	-	-	25/135/178	18/66/82	0.33
ADNI GO/2 (n=631)	**(n=129)**	**(n=40)**	**(n=242)**	**(n=122)**	**(n=98)**	**Diff *p***
WMHv* (SD)	6.8 (13)	8.5 (11)	7.3 (9)	7.7 (10)	8.7 (10)	0.7
CI (-/+)[a]	6+,113-,10 NA	40 NA	20+, 184-, 38 NA	6+, 75-, 41 NA	1+, 35-, 52 NA	0.35
Age, Y (SD)	74 (6)	72 (5)	71 (7)	72 (8)	75 (8)	0.0002
Sex (F/M)	62 F, 67 M	27 F, 13 M	102 F, 140 M	52 F, 70 M	38 F, 60 M	0.025
BP (systolic)	134 (16)	131 (17)	132 (17)	132 (18)	131 (17)	0.76
BP (diastolic)	74 (10)	72 (9)	73 (9)	73 (10)	74 (10)	0.87
MMSE (SD)	29 (1)	29 (1)	28 (2)	28 (2)	23 (2)	<0.0001
Education, Y (SD)	16 (3)	17 (3)	16 (3)	16 (3)	16 (3)	0.16
***APOE* ε4 status (-/+)**	34+,95-	12+,28-	105+,137-	69+,53-	66+,32-	<0.0001
rs6971 genotype (LAB/MAB/HAB)	19/51/59	5/20/15	26/108/108	9/56/57	10/42/46	0.75

Note: *volumes are in mm^3, normalized to a standard space. [a]Sample sizes for MRI cerebral infarcts (CI) are different than those indicated in column headers, some non-overlapping subjects were evaluated for CI: ADNI1

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(n_{CN}=196, n_{LMCI}=339, n_{AD}=167), ADNI GO/2 (n_{CN}=123, n_{EMCI}=207,n_{LMCI}=83,n_{AD}=36). Continuous variables (age, education, MMSE, and BP) were analyzed for diagnosis group differences using ANOVA (two-tailed). Factor variables (sex, CI, *APOE* ε4 status, and rs6971 genotype) were analyzed using Fisher's exact test (two-tailed). LAB=low affinity binders (rs6971 A/A); MAB=medium affinity binders (rs6971 A/G); HAB=high affinity binders (rs6971 G/G); MAF=minor allele frequency; WMHv=white matter hyperintensity volume; CI = cerebral infarcts detected by MRI; MMSE=mini mental status examination; APOE=Apolipoprotein E; BP=blood pressure (mmHg); MMSE=mini mental status exam; SD=standard deviation; M=male; F=female; Y=years; CN=cognitively normal; SMC=some memory concern; EMCI=early mild cognitive impairment; LMCI=late mild cognitive impairment; AD=Alzheimer's disease.

In the ADNI 1, GO, and 2 combined Caucasian sample (n=1 151), rs6971 genotype was not associated with the presence of cerebral infarcts (Wald X^2_1=1.98, p_{raw}=0.16) (Figure 5-1). However, there was a nominal association with infarcts in the LMCI group (Wald X^2_1=3.98, p_{raw}=0.047), whereby likelihood of having infarcts increased stepwise with decreasing binding affinity ($OR_{LAB:HAB}$=2.97, $C.I._{95\%}$=[1.01,8.70]). No associations were observed in the CN (Wald X^2_1=2.67, p_{raw}=0.10), EMCI (Wald X^2_1=0.01, p_{raw}=0.94) or Alzheimer's disease group (Wald X^2_1=1.13, p_{raw}=0.29), though there were no Alzheimer's disease subjects who had both infarcts and the rs6971 LAB genotype, making additive model inference in this subgroup impossible.

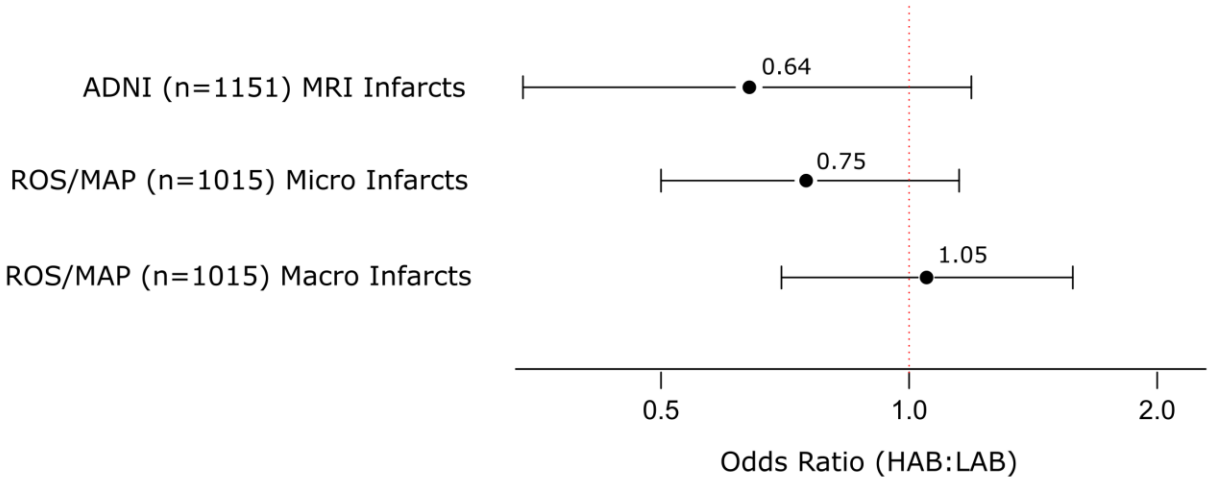

Figure 5-1. Odds Ratios (ORs) for rs6971 effect on presence of cerebral infarcts in the ADNI 1, GO and 2 combined sample (n=1 151) and ROS/MAP (n=1 015) samples. ORs are shown for the additive model homozygote contrast (i.e. HABs vs. LABs). ROS/MAP=Religious Orders Study/Memory and Aging Project; ADNI=Alzheimer's

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Disease Neuroimaging Initiative; HAB=high affinity binding genotype; MAB=medium affinity binding genotype; LAB=low affinity binding genotype.

In three independent samples (ADNI 1, ADNI GO/2, and ROS/MAP) there were no associations of rs6971 genotype with WMHv (Figure 5-2), though in ADNI 1 (n=699), rs6971 genotype showed a trend-level association with WMHv (additive $F_{1,692}$=2.87, p_{raw}=0.091). The direction of this trend in ADNI 1 was consistent with that observed in the combined sample for infarcts, with HAB subjects showing both lower risk for presence of infarcts and greater WMHv (though neither result was significant at p<0.05). Also, in ADNI GO an 2 (n=631), there was a weak association of rs6971 with WMHv in the EMCI group (n=242, additive $F_{1,234}$=3.55, p_{raw}=0.061), however the allelic pattern of this trend was in the opposite direction as in ADNI 1, whereby LAB subjects had lower WMHv. No interactions of rs6971 genotype and *APOE* ε4 status were found (all p_{raw}>0.1).

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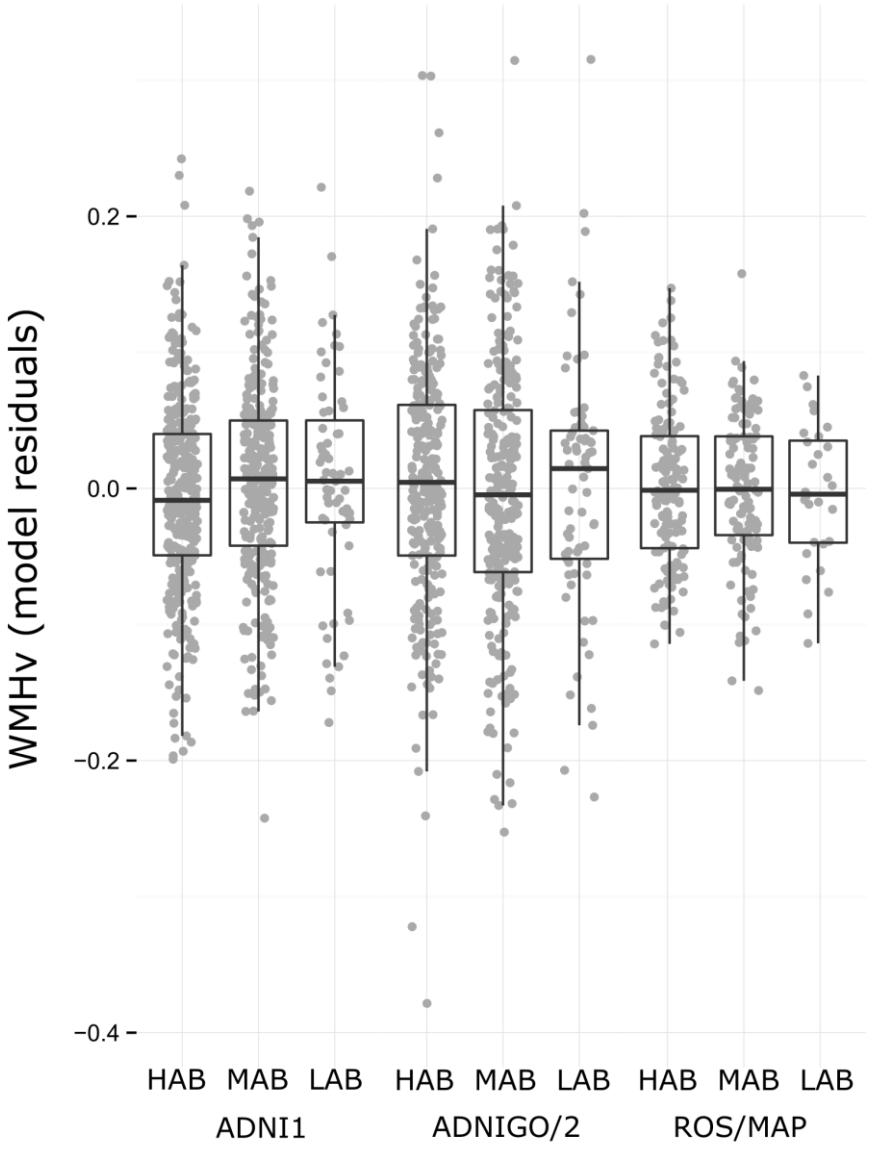

Figure 5-2. White matter hyperintensity volume (WMHv) plotted by rs6971 genotype group in ADNI 1 (n=699), ADNI GO and 2 (n=631), and ROS/MAP (n=291) samples. WMHv is plotted as the residuals of linear models that include the following co-variates: age, sex, diastolic blood pressure (hypertension y/n for ROS/MAP), *APOE* ε4 status, education, and total intracranial volume (ADNI 2 only). No results show evidence to reject null association (all additive P>0.05). ROS/MAP=Religious Orders Study/Memory and Aging Project; ADNI=Alzheimer's Disease Neuroimaging Initiative; HAB=high affinity binding genotype; MAB=medium affinity binding genotype; LAB=low affinity binding genotype.

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5.4.2 Plasma Inflammatory Biomarkers (ADNI and ROS/MAP)

In the ADNI 1 subsample with plasma inflammatory biomarker data (n=520), no main effect of rs6971 genotype was found for any biomarker (all p_{raw}>0.1, see Figure 5-3). Post-hoc testing revealed effects of genotype on TNFα levels in the Alzheimer's disease subjects (n=104, additive $F_{1,99}$=10.92, p_{raw}=0.0013), however this effect was not replicated in the ROS/MAP Alzheimer's disease subsample (n=85, additive $F_{1,80}$=0.34, p=0.24). Further, there were no main effects of rs6971 genotype on any biomarker in the ROS/MAP sample subset (Figure 5-3) or within any diagnostic group separately, and no significant interactions of rs6971 and *APOE* ε4 status were found in either subsample (all p_{cor}>0.05).

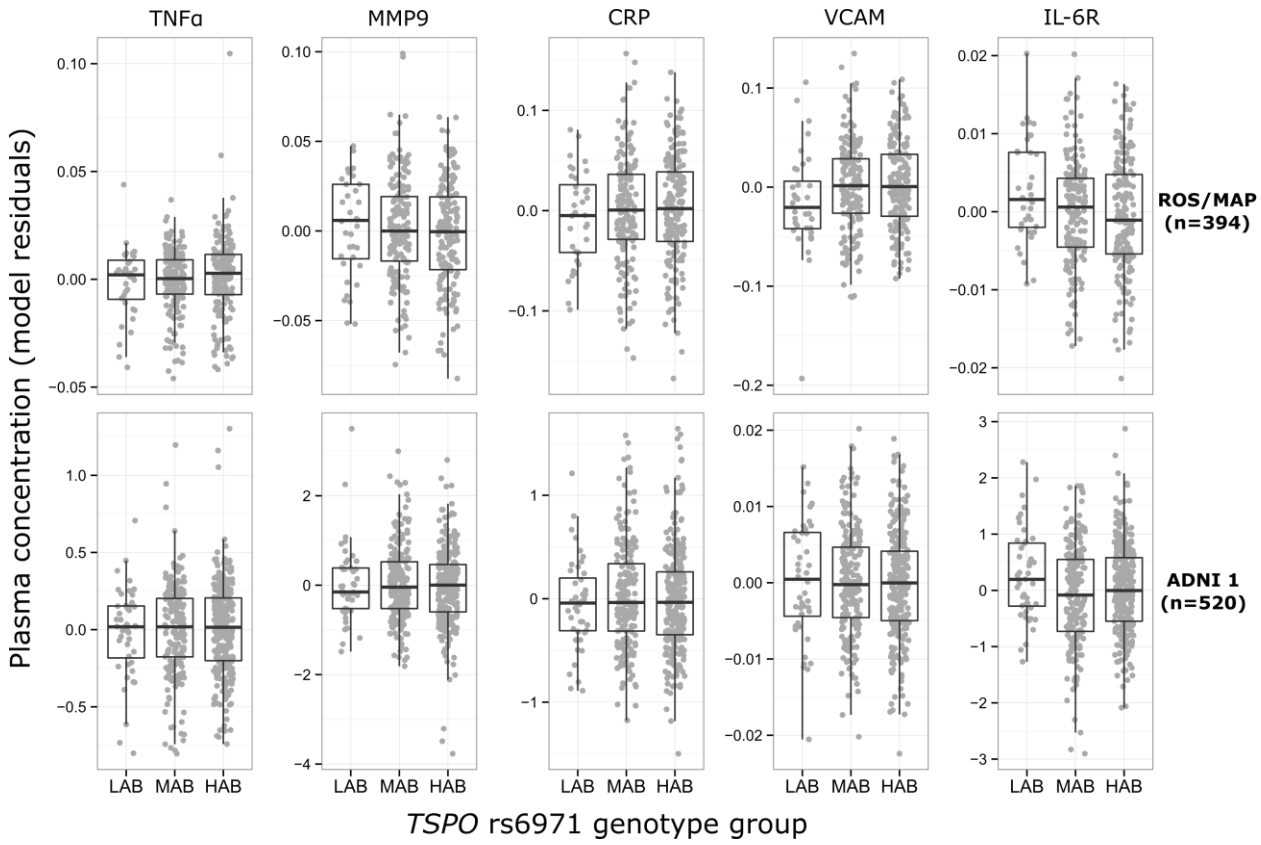

Figure 5-3. Null effects of *TSPO* rs6971 genotype on plasma inflammatory biomarkers in the ROS/MAP (n=394) and ADNI 1 (n=520) subsamples. All two-sided *P*-values are >0.4 after within-sample Bonferroni correction for five comparisons. Plotted residuals on y-axes are from models co-varying for age, sex, clinical diagnosis, and *APOE* ε4 status.

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5.4.3 Postmortem Neuropathology and Microglial Activation (ROS/MAP)

In the whole sample (n=1 015), rs6971 was not associated with macro or micro cerebral infarcts (all p_{raw}>0.1, see Figure 5-1). No main effects of rs6971 genotype were found for degree of CAA (n=897, additive Wald X^2_1=0.12, p_{raw} =0.73) (Figure 5-4). Finally, we found no associations of rs6971 with either the density of active microglia at any stage of activation, nor the ratio of highly active microglia to relatively inactive microglia, in any region tested (all p_{raw}>0.05, see Figure 5-5). Interaction analyses revealed no interactions of rs6971 and *APOE* ε4 status on any postmortem pathological measure (all p_{raw}>0.1).

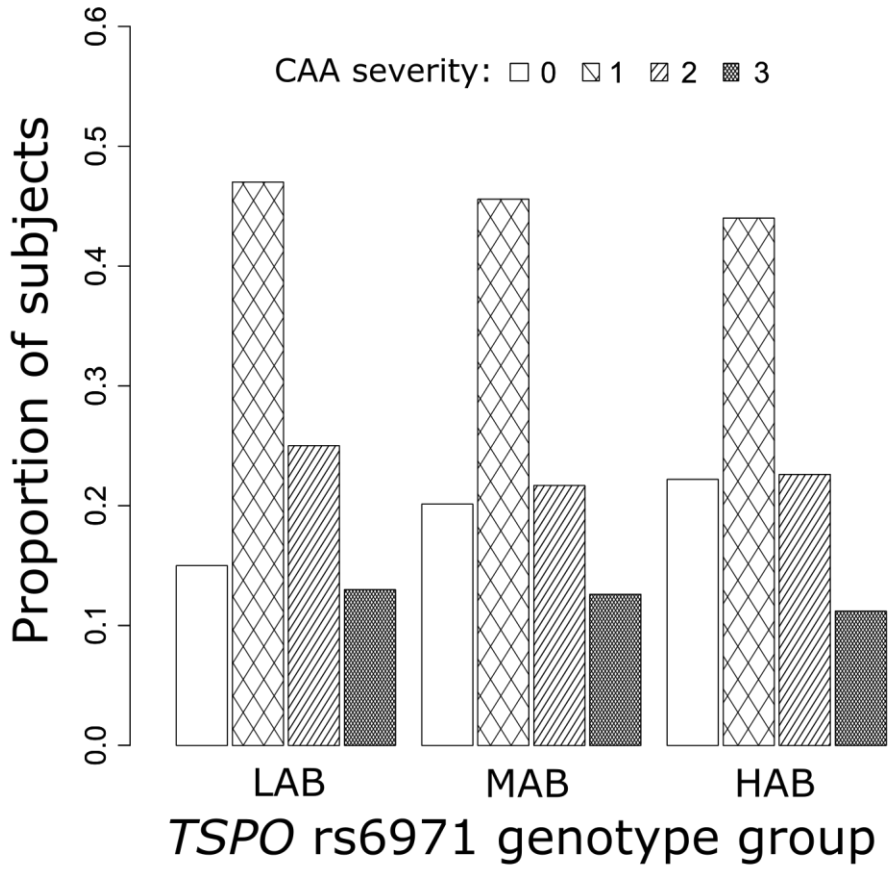

Figure 5-4. Null association of *TSPO* rs6971 genotype with severity of CAA in the ROS/MAP post-mortem dataset (n=1 015). Data shown are raw proportions of subjects in each genotype group with varying degrees of CAA severity. CAA = cerebral amyloid angiopathy.

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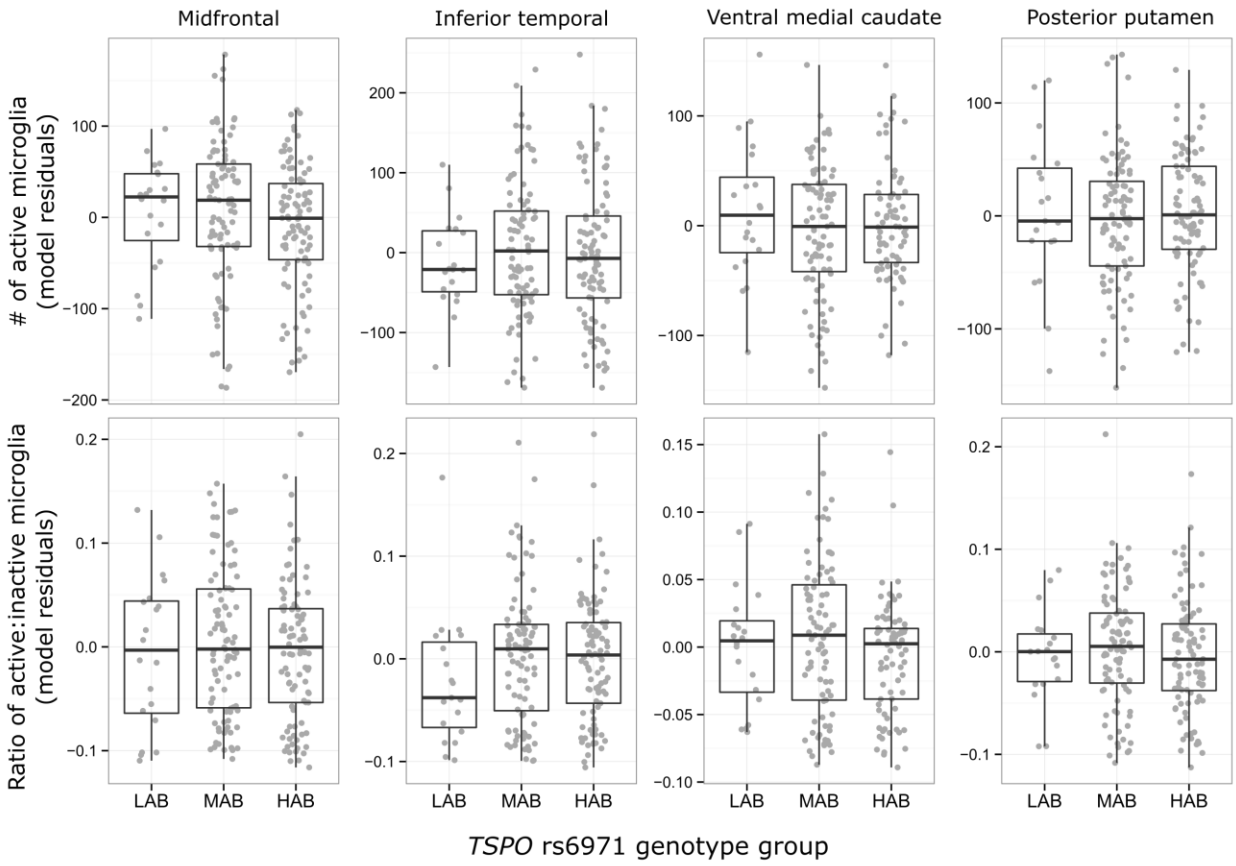

Figure 5-5. Null associations of *TSPO* rs6971 genotype with microglial activation in a subset of the ROS/MAP post-mortem dataset (n=209). All two-sided *P*-values are equal to 1 after Bonferroni correction for eight comparisons. Ratios in four bottom plots were transformed using square root transformations.

5.5 Discussion

Our study is the first to analyze variation in *TSPO* (rs6971) with respect to imaging and plasma biomarkers related to neuroinflammation in both Alzheimer's disease patients and cognitively normal elderly subjects. To our knowledge, we are also the first to analyze the effect of rs6971 on microglial activation and postmortem neuropathology. In a large sample of postmortem brains from elderly subjects, we found no significant effects of *TSPO* genotype with respect to postmortem or *in vivo* cerebral infarcts (Figure 5-1). While we found a marginally protective effect ($0.5<p<0.1$) of genotype on *in vivo* WMH burden (ADNI 1), this finding did not replicate in either of two independent samples (ADNI GO and 2, ROS/MAP) (Figure 5-2). Further, we found diagnosis-dependent effects of *TSPO* genotype on plasma levels of TNFα (ADNI 1) that

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did not replicate in an independent sample (ROS/MAP). We found no significant association of *TSPO* genotype with either degree of cerebral amyloid angiopathy or microglial activation in postmortem tissue (Figures 5-4 and 5-5). Exploratory analyses found a potential interaction of *TSPO* and *APOE* genotypes, finding that *APOE* ε4 status may modulate an effect of *TSPO* genotype on macro cerebral infarcts.

Our hypothesis specified that genotype-driven alterations in TSPO structure may influence its downstream neuroprotective action (Ferzaz et al., 2002; Girard et al., 2008; Ryu et al., 2005; Veiga et al., 2005), thereby conferring early and enduring risk across the lifespan for atherosclerotic damage, SVD, and potentially inflammatory dysregulation. The observed trend effects of TSPO genotype on vascular phenotypes (as well as amyloid angiopathy) were most prominent in the CN subgroups of both samples; this suggests that the effects of TSPO variation may be dependent on the time course of illness, with the greatest genotype differences observed in pre-symptomatic or early stage AD subjects. Interestingly, the HAB subjects appeared to be marginally protected from micro infarcts in the ROS/MAP sample, there was no observed effect of genotype on CAA. While association between micro infarcts and severe CAA has been previously shown (Soontornniyomkij et al., 2010), it is possible that the level of neuropathology across diagnostic groups in the ROS/MAP sample is not high enough to observe a similar pattern of *TSPO* effect for CAA as micro infarcts; the results are unaffected by co-varying for CAA. Due to uniquely resilient characteristics of the ROS/MAP sample (ROS study consisting exclusively of priests, nuns and brothers with well-documented healthy lifestyles and greater than average lifespan (Negash et al., 2011)), it may be that protective environmental factors associated with lifestyle have facilitated efficient amyloid clearance, but have not influenced amyloid-independent inflammatory processes associated with micro infarction to the same degree. Also, a recent review analyzed studies of *in vivo* neuroinflammation and amyloid deposition, as measured by PET, and found no correlation between individual levels of inflammation and amyloid (Hommet et al., 2014).

In our ADNI 1 analysis of inflammatory biomarkers, we found diagnosis-dependent effects of *TSPO* genotype on peripheral levels of TNFα, whereby HABs had higher levels if they were also diagnosed with AD. TNFα is generally regarded as a potent "traditional" pro-inflammatory cytokine associated with the innate immune response, but recent evidence suggests that TNFα may have regulatory roles in a diverse set of processes within the central nervous system

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including cell viability, synaptic plasticity, and learning and memory (Frankola, Greig, Luo, & Tweedie, 2011; Olmos & Llado, 2014). This finding, however, was not successfully replicated in a second independent group of subjects, suggesting that the association is likely null. This lack of association is further supported by our negative observations for the other four biomarkers analyzed, including CRP.

In line with our primarily null findings, the existing literature on TSPO function is somewhat equivocal. Our results align with a very recent investigation of *TSPO* genotype effects on *in vivo* amyloid and cognition in the ADNI cohort (Fan et al., 2015), which found no differences between HAB, MAB, and LAB subjects across or within diagnostic subgroups separately. At the molecular level, the role of TSPO in steroidogenesis has recently been called into question (Stocco, 2014) following two studies: one showing that testicular production of testosterone was unaltered in a conditional TSPO knockout mouse (Morohaku et al., 2014), and the other showing that the global knockout mouse was viable (contrary to previous reports (Papadopoulos et al., 1997)) and showed no differences in expression of genes related to steroidogensis (Tu et al., 2014). It could be argued, however, that these studies do not preclude the possibility that when present, different forms of TSPO (perhaps resulting from genetic variation) may function in potentially divergent ways. To test this directly in the context of immune system activation, we examined the effect of genotype on microglial activation in human postmortem brain tissue, finding no genotypic group differences in densities of active microglia or in the ratio of highly active to relatively inactive microglia. Importantly, these results suggest that the mechanism via which *TSPO* genotype may influence downstream pathology and disease risk (not just restricted to neurodegenerative disorders) does not involve the modulation of microglial activation.

Importantly, the immune response is neither purely damaging nor protective. Both M1- and M2-like microglial phenotypes (with pro- and anti-inflammatory roles, respectively (Mantovani, Biswas, Galdiero, Sica, & Locati, 2013; Prinz, Priller, Sisodia, & Ransohoff, 2011)), each with unique gene expression profiles (Butovsky et al., 2013), have been shown to have distinct and often opposing roles in disease (Goldmann & Prinz, 2013; Saijo & Glass, 2011). For example, it has been observed that both pro- and anti-inflammatory cytokines expressed by activated microglia have divergent effects on amyloid production and cell death in the hippocampus (Chakrabarty et al., 2011). The microglial response in mice is different at different ages (characterized by significant changes in expression profiles) (Crain, Nikodemova, & Watters,

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2013), demonstrating how a changing physiological environment can influence the response of identical stimuli to result in both M1- and M2-like microglial responses. Given that we did not quantify the relative densities of M1- and M2-like microglia in our brain tissue samples, it is possible that a genotype effect on either specific cellular phenotype may have been missed.

There are several additional limitations to this study. First, while efforts were made to ensure genotype groups were matched for important socio-demographic factors, potential confounding effects of unaccounted for subclinical pathology or other environmental factors cannot be ruled out. Second, population stratification due to ethnic diversity is a concern in any genetic analysis and must be considered as a potential confounder in our study. The observed MAFs for *TSPO* rs6971 in the ROS/MAP and ADNI samples were 0.32 and 0.31, respectively, consistent with observations in other Caucasian populations (HapMap-CEU MAF=0.29 (http://hapmap.ncbi.nlm.nih.gov/cgi-perl/snp_details_phase3?name=rs6971&source=hapmap28_B36&tmpl=snp_details_phase3)). The similarity of our genotype frequencies with those observed in large, homogenous population studies should serve to reduce concerns over ethnic heterogeneity within samples. Third, while we believe that some inference can be made as to the temporal pattern of genotype effect in our samples based on current diagnosis, we are aware of the correlational nature of cross-sectional, observational studies and are unable to infer causation regarding *TSPO* variation and Alzheimer's disease risk phenotypes. Fourth, we recognize that the literature shows discordance between patterns of inflammatory biomarkers measured in serum vs. CSF (Swardfager et al., 2010), and thus our results (obtained from plasma aliquots) may not rule out brain-specific inflammation. Finally, study design differences between ADNI and ROS/MAP, particularly in sample recruitment and data collection, should be acknowledged when considering our findings. While inter-study variability in image processing pipelines (for WMHv) and plasma protein quantification methods could potentially drive discordance between sample results, the fact that we found no significant effects of genotype in either sample (other than for plasma TNFα in ADNI Alzheimer's disease subjects only) should alleviate concerns regarding false negatives.

In conclusion, our results find no evidence for genetic variation in *TSPO* as a cerebrovascular and inflammatory risk factor related to neurodegeneration. In particular, the observed null effects of rs6971 on levels of microglial density across brain regions suggest that TSPO structural differences due to genotype likely do not interact with endogenous ligands in a manner that

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influences microglial activation. Our findings echo the recent discordance between functional studies of TSPO that have brought about controversy regarding its importance in embryonic development, steroidogenesis, and mitochondrial permeability (Li, Liu, Garavito, & Ferguson-Miller, 2015). While we provide the first insight into the potential effects of rs6971 variation on structural imaging and postmortem brain pathology in humans, continued *in vitro* and animal model experiments of differential TSPO binding would be required to verify the pathway-level impact of this variation with respect to Alzheimer's disease etiopathology.

5.6 Acknowledgements

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Chapter 6

6 Genetic Interaction between *SORL1* and *BDNF* Regulates Isoform-Specific *SORL1* Expression and Brain Amyloid

Felsky D et al. Genetic Interaction between *SORL1* and *BDNF* Regulates Isoform-Specific *SORL1* Regulation and Influences Brain Amyloid.

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6.1 Abstract

Background: Variants within the sortilin-like receptor (*SORL1*) gene are replicated at genome-wide significance for Alzheimer's disease (AD) risk; however, their mechanisms of effect are largely unknown. SORL1 acts within both amyloidogenic and vascular pathways, but its mRNA is also upregulated by the brain-derived neurotrophic factor (BDNF), in a *SORL1* genotype-dependent manner. The *BDNF* Val66Met variant affects the cellular secretion of BDNF and may therefore interact with *SORL1* genotypes to influence *SORL1* expression and downstream AD-related phenotypes. Importantly, *SORL1* transcript isoforms may be preferentially affected by these interactions and play unique roles in AD-related brain changes.

Methods: This study included data from n=608 subjects from the Religious Orders Study/Memory and Aging Project (ROS/MAP), and n=1 285 subjects from the Alzheimer's Disease Neuroimaging Initiative (ADNI phases 1, GO, and 2). In the ROS/MAP sample, 10 *SORL1* transcripts were quantified as expressed vs. not expressed in prefrontal cortex of 441 postmortem brain samples using RNA-sequencing. For all transcripts, the interaction between each common SNP within 10kb of the *SORL1* locus and *BDNF* Val66Met was tested using logistic regression. Interactions showing significance at $p<0.05$ after locus-wide multiple testing correction were carried forward for further analyses on *in vivo* amyloid (measured with [18F]Florbetapir PET) in ADNI (n=710), entorhinal cortex volume in ROS/MAP (n=172) and ADNI (n=1 285), and white matter tract fractional anisotropy in ADNI 2 (n=185).

Results: In the ROS/MAP sample isoform expression analyses, 36 tests survived correction for multiple testing. All 36 models were for the same transcript, SORL1-005, a putative truncated protein-coding transcript of 1 124 amino acids, and all SNPs were in high LD (top SNP rs12364988, $p=1.25\text{x}10^{-5}$). The rs12364988^{T} allele reduced likelihood of SORL1-005 expression in the $BDNF^{Val}$ homozygotes, but greatly increased likelihood of expression among $BDNF^{Met}$ carriers. This effect was driven entirely by a subsample of non-pathological AD subjects (n=179). Further, increased SORL1-005 was weakly associated with increased midfrontal diffuse plaque pathology in the ROS/MAP sample ($F_{1,431}=4.07$, $p=0.044$). In the ADNI [18F]Florbetapir PET imaging sample (n=710), *SORL1-BDNF* interactions from expression analyses also significantly influenced Aβ load in multiple frontal ROIs (top SNP rs618874, $F_{1,649}=12.12$, $p=5.3\text{x}10^{-4}$) such that genotype groups more likely to express SORL1-005 also showed higher

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Aβ. Finally, *SORL1-BDNF* interactions also influenced entorhinal cortex volumes in ROS/MAP ($F_{1,161}$=6.64 ,*p*=0.011, n=172) and ADNI ($F_{1,1268}$=4.69, *p*=0.031, n=1 235), as well as white matter fractional anisotropy in ADNI 2 ($F_{1,172}$=6.44, *p*=0.012, n=185), though all at sub-threshold significance.

Conclusions: Our findings point toward a novel interaction between *SORL1* and *BDNF* variants that may be important for regulating isoform-specific *SORL1* expression in non-AD subjects. The top interacting *SORL1* SNP found in locus-wide analyses, rs12364988, is part of the same haplotype block recently found to determine BDNF-dependent upregulation of *SORL1* mRNA in neurons derived from human induced pluripotent stem cells. *SORL1-BDNF* genetic interactions also predicted differences in diffuse plaques postmortem and total *in vivo* amyloid burden, as well as AD-vulnerable brain structure across two independent samples. The effects of this gene-gene interaction suggest a possible mechanistic role of the SORL1-005 transcript in brain changes related to non-pathological aging, and may help bridge the gap between AD risk processes and those associated with resilience in healthy aging.

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6.2 Introduction

Variants within the sortilin-related receptor (SORL1, SORLA, LR11) gene are among the most highly-replicated genetic risk factors for late-onset Alzheimer's disease (AD); they have been associated with AD diagnosis in candidate studies (Rogaeva et al., 2007), independent genome-wide association studies (Lambert et al., 2013; Miyashita et al., 2013), and meta-analyses (Lambert et al., 2013; Reitz et al., 2011). SORL1 plays important roles in amyloid processing and trafficking, functioning to recycle APP to the neuronal membrane (Andersen et al., 2005) as well as to directly target Aβ toward lysosomal cellular compartments (Caglayan et al., 2014). While some studies have implicated *SORL1* genotypes independently in gene expression (Caglayan et al., 2012, p. 1; McCarthy et al., 2012), the transcriptional control of *SORL1* likely depends on extragenous factors, particularly levels of the brain-derived neurotrophic factor (BDNF) (Rohe et al., 2009), as well as *SORL1* genotype. Accordingly, it was recently shown that BDNF administration in iPSC-derived neuron cultures up-regulate SORL1 expression in a *SORL1*-genotype dependent manner (Young et al., 2015). Studying the interaction of functional *BDNF* and *SORL1* genotypes in large, well-characterized samples may provide more insight into the nature of this transcriptional regulatory mechanism and risk vs. resilience mechanisms for AD.

The *BDNF* Val66Met polymorphism determines the activity-dependent secretion of BDNF (Egan et al., 2003), with Met<Val, and also the function of the BDNF pro-peptide in facilitating hippocampal long-term depression (LTD) (Mizui et al., 2015), with the Met allele significantly diminishing LTD. In this way, *BDNF* Val66Met may serve as a functional assay for BDNF activity in the brain. We and others have demonstrated effects of *BDNF* Val66Met on brain structure (Toro et al., 2009; Voineskos et al., 2011; C. Wang et al., 2014), function (Cárdenas-Morales, Grön, Sim, Stingl, & Kammer, 2014; Lisiecka et al., 2015), and cognition (Dincheva et al., 2012; S. E. Harris et al., 2006), related to risk for AD. In addition, we have shown effects of *BDNF* Val66Met on temporal white matter tract integrity as measured with diffusion tensor imaging (Voineskos et al., 2011), which is highly predictive of conversion from cognitively normal (CN) to amnestic mild cognitive impairment (MCI) (Zhuang et al., 2012). These effects may be downstream consequences of BDNF's stimulation of SORL1 activity (Rohe et al., 2013),

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and therefore may be subject to modulation by both *BDNF* and *SORL1* genotypes interdependently.

We have previously shown a main effect of *SORL1* genotype on levels of prefrontal *SORL1* mRNA in postmortem brain at early stages of the human lifespan (Felsky et al., 2014), however, the microarray technology used in that study was unable to capture transcript diversity, and previous reports have shown differential SORL1 isoform expression both in AD (Grear et al., 2009, p. 1) and as a result of *SORL1* genotype (Caglayan et al., 2012). The *SORL1* gene contains 44 exons and the ensembl database (http://useast.ensembl.org) currently lists 13 distinct SORL1 transcripts. RNA-sequencing (RNA-seq) technology offers distinct advantages over probe-based methodologies as it allows for the alignment of assembled transcript reads to any sequence template and the estimation of isoform expression based on these reads. We have also previously shown age-dependent effects of the *BDNF* Val66Met polymorphism on white matter microstructure, cortical thickness, and episodic memory performance in healthy adults (Voineskos et al., 2011), suggesting that as-of-yet unidentified factors may act to influence *BDNF*'s protective effects on neurodegeneration and cognitive aging.

Given the regulatory interaction of BDNF protein with *SORL1* genotype in human iPSC-derived neurons (Young et al., 2015), we hypothesized that common *SORL1* gene variants may interact with *BDNF* Val66Met to influence the expression of *SORL1* transcripts. Further, given the functions of SORL1 within the amyloidogenic cascade, we hypothesized that SNP-SNP interactions predicting altered *SORL1* expression may affect amyloid neuropathology as well as brain structures at risk in the early stages of AD, such as entorhinal cortex (Bobinski et al., 1999; Gómez-Isla et al., 1996; J. L. Price et al., 2001) and fronto-temporal white matter (Selnes et al., 2013; Zhuang et al., 2012). To test this, we performed an unbiased locus-wide gene-gene interaction analysis of *SORL1* SNPs with *BDNF* Val66Met to model the expression of 10 *SORL1* transcripts, identified by RNA-seq of postmortem prefrontal cortex tissue, in a large sample (n=441) of subjects from the Religious Orders Study and Memory and Aging Project (ROS/MAP). Transcripts showing significant evidence for regulation by *SORL1-BDNF* interactions were then tested for effects on postmortem frontal lobe amyloid deposition in ROS/MAP. We then tested significant SNP-SNP interactions for effects on *in vivo* frontal amyloid load, as measured by [18F]Florbetapir PET, in 710 subjects from the Alzheimer's Disease Neuroimaging Initiative (ADNI). Finally, to explore potential downstream effects of

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these SNP-SNP interactions on brain structure, we examined 1 285 subjects from ROS/MAP and 172 subjects from ADNI with MRI estimates of entorhinal cortex volume, and 185 subjects from ADNI 2 with diffusion tensor imaging (DTI) data for tracts implicated in AD.

6.3 Methods

6.3.1 Religious Orders Study and Memory and Aging Project (ROS/MAP)

6.3.1.1 Study Participants

A total of 441 subjects with genomic, RNA sequencing, and neuropathological data were included in the present study. All participants were from the Religious Orders Study (ROS) (Bennett, Schneider, Arvanitakis, et al., 2012) and Memory and Aging Project (MAP) (Bennett, Schneider, Arvanitakis, et al., 2012); two large ongoing cohort studies based out of the Rush Alzheimer's Disease Center at Rush University in Chicago, IL. ROS began in 1994 recruiting brothers, nuns, and priests over the age of 53 – all healthy at time of study entry – requiring longitudinal clinical, cognitive and biometric assessments, as well as agreement to donate brain for autopsy at time of death. MAP began in 1998 according to the same study design, but with a more general target population for sampling; subjects aged 55 and over were recruited from the general elderly population of the Chicago area, rather than exclusively from members of clergy. All subjects were assessed with a comprehensive decision tree algorithm as well as a uniform, structured, clinical evaluation that included a self-report medical history obtained by trained nurses and research technicians, a neurologic examination by trained nurses and cognitive function testing by trained neuropsychological test technicians. Both studies were approved by the Institutional Review Board of Rush University Medical Center and enroll older persons without dementia who agree to annual evaluation and autopsy. The follow-up rates for both studies exceed 90% and autopsy rates exceed 80%.

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6.3.1.2 Genetics

Genotyping of all subjects was performed using the Affymetrix (Santa Clara, CA, USA) Genechip 6.0 platform. *APOE* (rs7412 and rs429358) genotypes were imputed from MACH (version 1.0.16a) and HapMap release 22 CEU (build 36), as previously described (Chibnik et al., 2011). Common variants within 10kb of the *SORL1* locus (chromosome 11, position 121 312 912 - 121 514 471, GRCh37 coordinates) were extracted using PLINK (v1.90b). A total of 327 high confidence imputed SNPs in this region were pruned for minor allele frequency (MAF>0.1) and Hardy-Weinberg Equilibrium (HWE p>0.001), resulting in a final set of 160 variants for analysis. Pairwise linkage was assessed in this set of 160 SNPs to determine the number of independent tests locus-wide, as outlined in Statistical Analysis (6.3.3).

6.3.1.3 Postmortem SORL1 Isoform Expression

RNA-Seq expression data were generated from frozen dorsolateral prefrontal cortex tissues following the construction of complementary DNA libraries, as previously published (Yu, Chibnik, et al., 2015). Briefly, RNA was extracted using Qiagen's miRNeasey mini kit and the RNase free DNase Set. Samples were quantified by Nanodrop and quality evaluated by Agilent Bioanalyzer. RNA-Seq library was prepared on Broad Institute's Genomics Platform using strand specific dUTP method (Levin et al., 2010) with poly-A selection (Adiconis et al., 2013). Samples were sequenced using the Illumina HiSeq platform to a depth of 50 million paired-end reads of 101 bp each. The paired-end reads were then mapped to SORL1 isoforms using the Ensemble human genome transcriptomic database (http://www.ensembl.org). Expression abundance was calculated as fragments per kilobase of exon per million reads mapped (FPKM).

6.3.1.4 Postmortem Neuropathology

A board-certified neuropathologist blinded to age and all clinical data established neuropathologic diagnoses for each subject based on scores from NIA-Reagan (NIA-Reagan, 1997), Braak (H. Braak & Braak, 1995), and CERAD (Mirra et al., 1991) classifications. Aβ and abnormal tau deposition in the frontal cortex was quanitified using immunohistochemistry and

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automated image processing for total amyloid and paired helical filament (PHF) tau in superior frontal cortex, and a modified Bielschowsky silver staining technique for neuritic and diffuse plaques, and neurofibrillary tangles (NFT) in midfrontal cortex, according to previously published methods (Bennett, Wilson, Boyle, et al., 2012). Quantitative scores for Aβ and PHFtau deposition (percent area occupied) and neuritic and diffuse plaques and NFTs (density by number/mm^2 in region with highest density) were computed separately and square root-transformed before analysis, as in previous analyses of this dataset (Bradshaw et al., 2013; Yu, Chibnik, et al., 2015).

6.3.1.5 *In Vivo* Structural MRI

A subset of n=172 ROS/MAP subjects underwent structural neuroimaging protocols (overlap of n=5 with subjects from expression dataset). High-resolution T1-weighted anatomical scans were obtained using a 3D magnetization-prepared rapid acquisition gradient-echo (MPRAGE) sequence (echo-time (TE)=2.8 msec, repetition time (TR)=6.3 msec, preparation time=1000 msec, flip-angle=8°, field-of-view (FOV)=24 cm × 24 cm, 160 sagittal slices, slice thickness=1 mm, no gap, 224×192 image matrix reconstructed to 256×256, and two repetitions) (Arfanakis et al., 2013). Estimates of entorhinal cortex and whole brain volume (mm^3) were made using the open-sourced Freesurfer software package (http://surfer.nmr.mgh.harvard.edu).

6.3.2 Alzheimer's Disease Neuroimaging Initiative (ADNI)

6.3.2.1 Study Participants

The Alzheimer's Disease Neuroimaging Initiative (ADNI) is a multi-center collaboration established in 2003, in which elderly subjects at various stages of cognitive impairment are assessed longitudinally for multi-modal imaging and other AD-related biomarkers. All subjects are administered clinical evaluations at time of study enrollment by trained physicians as previously described (Petersen et al., 2010). Complete details regarding study protocols, inclusion/exclusion criteria, and data collection and availability can be found at http://www.adni-info.org. All participants provided written informed consent, and each site's institutional review

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board approved study protocols. ADNI was conducted in three phases (ADNI 1, GO, and 2), for which different study protocols were implemented; therefore, total sample sizes vary depending on phenotype. For the present study, a total of 1 285 subjects from ADNI 1, GO, and 2 (CN, EMCI, SMC, LMCI, and AD) had genomic and baseline structural brain imaging data for the entorhinal cortex available. For analyses of *in vivo* Aβ pathology, a subset of 710 subjects from ADNI 2 had genomic and baseline Aβ [^{18}F]Florbetapir PET imaging data available.

6.3.2.2 Genetics

All ADNI 1 subjects were genotyped using the Quad 610 BeadChip (Illumina Inc., San Diego, CA), and ADNI 2 subjects were genotyped using the HumanOmniExpress BeadChip (Illumina Inc., San Diego, CA). Genetic quality control was conducted using PLINK (v1.90b). After aligning the genetic data to the human assembly GRCh37/hg19 using UCSC's liftOver tool2, haplotypes were prephased using SHAPEIT3 v2.r790, and imputation was performed using IMPUTE2 (v2.3.1), with the 1000 Genomes Phase1 integrated haplotypes as the reference panel. All SNPs with an IMPUTE2 info score of less than 0.5 were excluded from further analyses. Additionally, SNPs with HWE p <0.001, and MAF <0.01 were excluded from further analyses. All 160 *SORL1* SNPs derived from ROS/MAP preprocessing - as well as *BDNF* Val66Met - were imputed with high confidence in the ADNI 1, GO, and 2 samples and available for analyses.

6.3.2.3 *In Vivo* Structural MRI and DTI

A total of 1 285 subjects from ADNI 1, 2, and GO underwent structural MRI protocols to generate estimates of entorhinal cortex volume. High resolution T1-weighted volumetric magnetization prepared rapid gradient echo sequences were acquired in the sagittal orientation. A proton density/T2-weighted fast spin echo sequence was acquired in the axial orientation. Sites included in the ADNI protocol were required to pass rigorous scanner validation tests and scan acquisitions for each subject included a fluid-filled phantom. Validation procedures have been

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previously documented (Clifford R. Jack et al., 2008). Entorhinal cortex and total intracranial volumes were estimated for each subject using FreeSurfer software (v4.3) (Fischl, 2012).

Diffusion-weighted images (DWI) were acquired for a subset of 185 subjects from ADNI 2 (256 × 256 matrix; voxel size: 2.7 × 2.7 × 2.7 mm^3; TR=9000 ms; scan time=9 min). 46 separate images were acquired for each DTI scan: 5 T2-weighted images with no diffusion sensitization (*B*0 images) and 41 diffusion-weighted images (*B*=1000 s/mm^2). This protocol was chosen to optimize the signal-to-noise ratio in a fixed scan time (Jahanshad et al., 2010), and all images were checked visually for quality assurance to exclude scans with excessive motion or other artifacts. To generate estimates of fractional anisotropy (FA) for specific white matter tracts, diffusion tensor volumes were first generated from eddy- and EPI-corrected DWI scans using *dtifit* (from FMRIB's Software Library (Jenkinson et al., 2012)) and used to generate FA maps for each subject. The FA maps were registered to the John Hopkins University (JHU) DTI atlas (Mori et al., 2008) using a mutual information based elastic registration algorithm (Leow et al., 2007). The resulting deformation fields were then applied to the JHU "Eve" white matter atlas labels, which were then used to mask specific white matter tracts and calculate average FA within each region of interest (ROI). Further details are described on the ADNI website (http://adni.bitbucket.org/docs/DTIROI/DTI-ADNI_Methods-Thompson-Oct2012.pdf).

6.3.2.4 *In Vivo* Aβ [18F]Florbetapir PET

Details of brain Aβ [^{18}F]Florbetapir PET imaging and preprocessing in ADNI have been described elsewhere (Landau et al., 2013). Briefly, each subject's structural T1-weighted MRI scan was co-registered to their [^{18}F]Florbetapir scan using SPM5 (http://www.fil.ion.ucl.ac.uk/spm). FreeSurfer (v4.5) (Fischl, 2012) was used to parcellate each T1-weighted scan into cortical subregions that were used to calculate [^{18}F]Florbetapir means within ROIs. Signal from [^{18}F]Florbetapir within cortical subregions were not standardized and so we co-varied for signal from a composite reference region comprised of cerebellum, brainstem/pons, and subcortical white matter in our analyses. Since RNA-sequencing in the ROS/MAP sample was performed on tissue from the prefrontal cortex, we maintained regional specificity by analyzing a set of frontal cortical ROIs averaged over the left and right hemispheres: lateral orbitofrontal, medial orbitofrontal, pars opercularis, pars orbitalis, pars

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triangularis, rostral middle frontal, and superior frontal cortices. Further details are available on the ADNI website (http://adni.bitbucket.org/docs/UCBERKELEYAV45/ADNI_AV45_Methods_JagustLab_01.20.15.pdf).

6.3.3 Statistical Analysis

Imputed genetic data were processed using PLINK (v1.90b) software (Purcell et al., 2007) and all regression analyses were performed using R (v3.1.1) statistical software (http://www.r-project.org/) (R Core Team, 2014). Haploview (v4.2) (Barrett, Fry, Maller, & Daly, 2005) was used for calculations of linkage disequilibrium (LD) structure. Based on lack of expression, data for three out of 13 transcripts were unfit for analysis as outcome measures: SORL1-003, SORL1-004, SORL1-007 (See Figure 6-2 for size and position of all transcripts). All other transcripts showed heavily right-skewed FPKM distributions (skewness ranging from 0.71-5.3, all D'Agostino test (D'Agostino, 1970) $p<1.3x10^{-5}$) - for several transcripts, a large portion of subjects showed zero expression - that could not be coerced to normal (using Box-Cox power transformations (Box & Cox, 1964)) and thus were evaluated as binary outcomes (expressed above 0 FPKM vs. 0 FPKM, or median split, where appropriate) using logistic regression. Each of 160 SNPs (MAF>0.1 and HWE $p>0.001$) within the *SORL1* locus were tested for interaction with *BDNF* Val66Met, with each transcript as outcome using logistic regression, co-varying for *APOE* ε4 status, sequencing batch, age at death, sex, study (ROS vs. MAP), RNA integrity number (Schroeder et al., 2006), postmortem interval, ribosomal bases, and the first three principal components of the genotype co-variance matrix analyzed using EIGENSTRAT (A. L. Price et al., 2006). Additive genetic models were assumed for *SORL1* variants and *BDNF* Val66Met genotypes were grouped according to a dominant model ($BDNF^{Val}$ homozygotes vs. $BDNF^{Met}$ carriers), due to the rarity of the homozygous $BDNF^{Met}$ genotype (13/441 ROS/MAP subjects, 48/1 285 ADNI 1, GO, and 2 subjects). *APOE* ε4 was controlled for to help isolate the genetic effects of *SORL1* and *BDNF* variants, given the strong impact of ε4 status on AD pathological and imaging phenotypes (discussed in Section 1.4.4).

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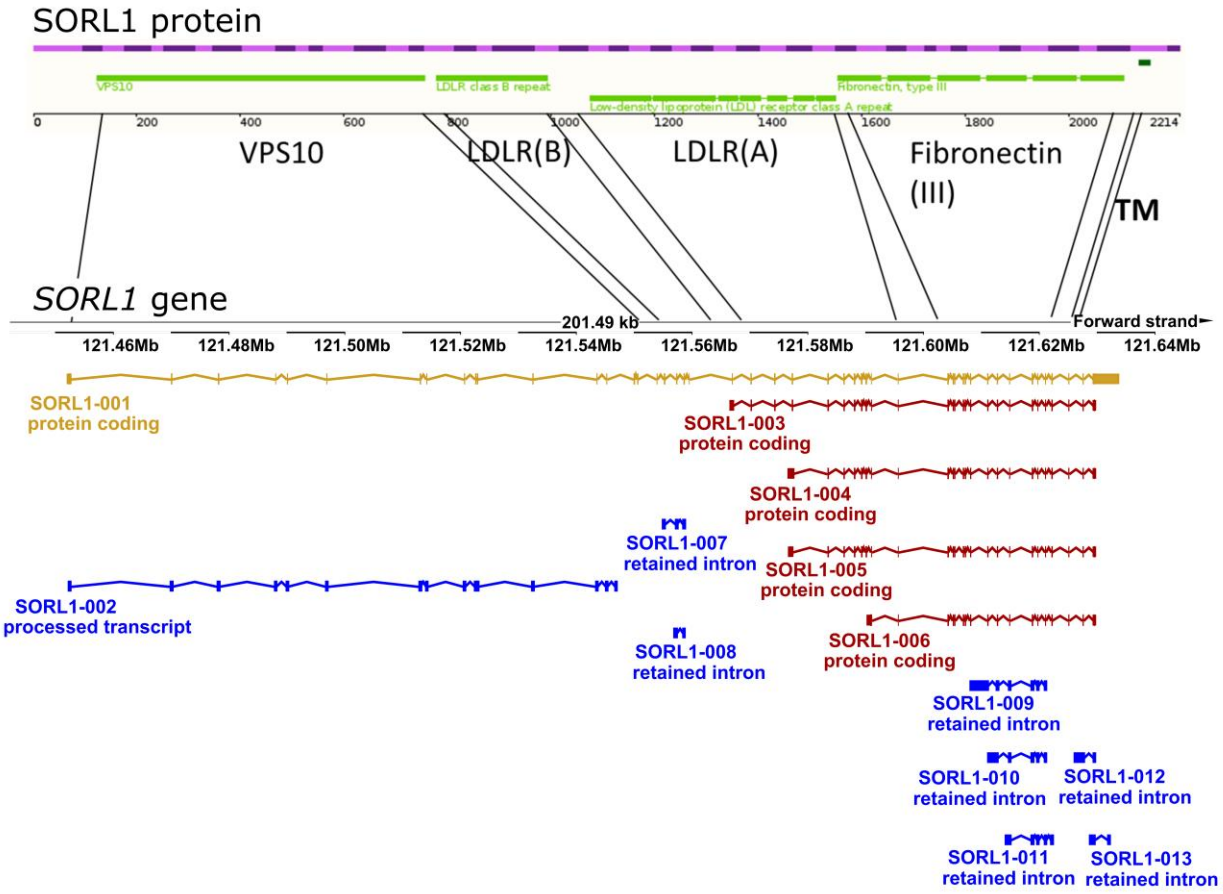

Figure 6-1. Diagram of *SORL1* transcripts and SORL1 protein domains (adapted from Ensembl database (http://www.ensembl.org), ENSG00000137642). All base pair positions are in GRCh38 assembly coordinates. Orange colour indicates merged Ensembl/Havana databse trasnscript, red indicates Ensemble protein coding transcript, and blue indicates non-protein coding transcripts. "Retained introns" are alternatively spliced transcripts that contain intronic sequence relative to other coding transcripts. "Processed transcripts" do not contain an open reading fram (ORF). VPS10 = vacuolar protein sorting 10; LDLR = low density lipoprotein receptor; TM = transmembrane.

To correct for multiple testing and account for LD structure across the tested SNPs, we first calculated the effective number of independent SNPs across the *SORL1* locus. This was accomplished by pruning imputed genotypes based on LD (window of 50 SNPs, a cutoff of r^2=0.2, and a step of 5 SNPs); of the 160 *SORL1* tested, six independent SNPs captured the haplotypic diversity at this locus (strong patterns of LD can be seen in Figure 6-1). Despite significant correlations observed between the expression levels of several of the ten *SORL1* transcripts analyzed, we treated each as an independent phenotype, resulting in a final

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experiment-wise Bonferroni corrected significance threshold of $p<8.33x10^{-4}$ (0.05 / 6 independent SNPs / 10 transcripts). To ascertain region-specific effects on postmortem neuropathology (RNA sequencing was performed on tissue from prefrontal cortex), transcripts that showed significant evidence for interactive regulation by *SORL1* variants and *BDNF* Val66Met were analyzed for effects mid-frontal neuritic plaques, diffuse plaques, and total amyloid in the ROS/MAP sample (n=440) using linear regression. Due to potential influence of RNA degradation on expression values, these analyses controlled for postmortem interval (PMI) and RIN, as well as age at death, sex, education, study (ROS vs. MAP), and *APOE* ε4 status.

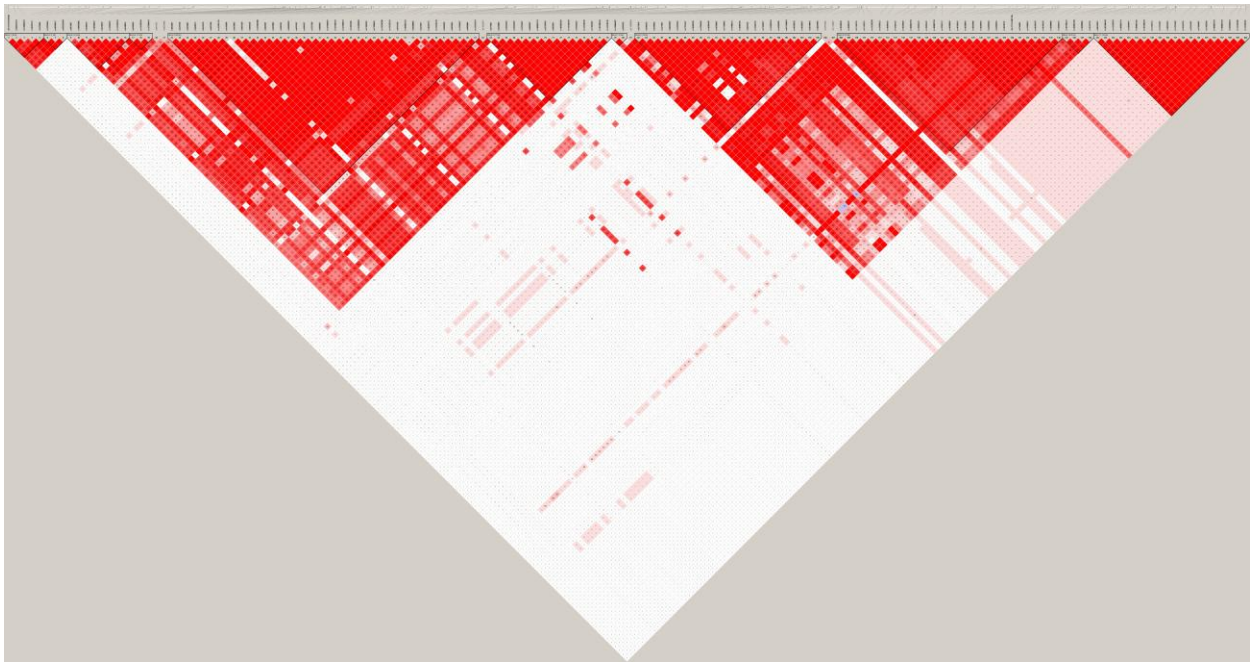

Figure 6-2. LD Structure of 160 *SORL1* variants analyzed in the ROS/MAP Sample (n=441). The region of interest was defined as the *SORL1* locus +/- 10kb (chr:11, pos. 121 452 203-121 633 693, GRCh38 coordinates).

SNP-SNP interactions from expression analyses that remained significant after correction for multiple testing were carried forward into the ADNI 2 sample to test for effects on *in vivo* Aβ [18F]Florbetapir PET (n=710). To maintain regional specificity, average amyloid load across seven frontal cortical ROIs were analyzed as outcomes for each gene-gene interaction using linear regression, co-varying for age, sex, education, ethnicity, diagnosis, *APOE* ε4 status,

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aggregate reference region signal, and ROI size. Interaction *p*-values were corrected for multiple testing using the FDR procedure (q=0.05) described by Benjamini and Hochberg (Benjamini & Hochberg, 1995).

Finally, to examine potential downstream consequences of altered amyloid pathology, the same set of SNP-SNP interactions identified by expression analyses were explored for effects on brain structure known to be vulnerable in the earliest stages of AD. In ROS/MAP and ADNI, subsamples of n=172 and n=1 285, respectively, had entorhinal cortex volumes available at time of analysis. Cortical volume was evaluated as outcome of linear regression models including each interaction, co-varying for sex, age, education, clinical diagnosis, *APOE* ε4 status, total brain volume, and either study (ROS vs. MAP) or phase (ADNI 1 vs. GO vs.2). Additionally, a subset of n=185 subjects from ADNI 2 who underwent DTI had average FA values for five bilateral fronto-temporal-occipital and interhemispheric white matter tracts implicated in AD: sagittal stratum (SS), hippocampal segment of cingulum bundle (CBH), splenium of corpus callosum (SCC), inferior fronto-occipital fascuiculus (IFO), and superior longitunial fasciculus (SLF). FA for each tract and SNP-SNP interaction was tested using linear regression, co-varying for age, sex, race, clinical diagnosis, and *APOE* ε4 status.

6.4 Results

6.4.1 SORL1 Transcript Expression and Postmortem Neuropathology

Sample demographics for ROS/MAP are summarized in Table 6-1. Out of 441 subjects included in the expression analyses, 262 had pathologically confirmed AD (59%). Of these 262 confirmed AD cases, 124 (47%) had a clinical diagnosis of AD, 62 (24%) had a diagnosis of MCI, 56 (21%) were cognitively normal, and 20 (8%) had additional other diagnoses when last assessed before death.

Table 6-1. ROS/MAP Sample Demographics

ROS/MAP expression (n=441)	**Non-AD (n=179)**	**PathoAD (n=179)**	**Diff (*p*)[1]**
Sex (F/M)	107 F, 72 M	171 F, 91 M	0.027

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Age at death (y(SD))	86.6 (7.2)	89.8 (5.9)	<0.0001		
Education (y(SD))	16.3 (3.6)	16.6 (3.4)	0.47		
MMSE (SD)	25.1 (6.8)	19.1 (9.4)	<0.0001		
RIN (SD)	7.2 (1.0)	7.1 (0.9)	0.21		
PMI (SD)	6.8 (4.1)	7.3 (5.4)	0.25		
***APOE* ε4 status (-/+)**	153-, 26+ (15%+)	168-, 94+ (36%+)	<0.0001		
***BDNF* genotype (valval/met carrier)**	116Val, 63M	170Val, 92M	1		
ROS/MAP MRI (n=172)	**CN (n=112)**	**MCI (n=41)**	**AD (n=13)**	**Other[2] (n=6)**	**Diff (*p*)[1]**
Sex (F/M)	81 F, 31 M	30 F, 11 M	12 F, 1 M	3 F, 3 M	0.25
Age at scan (y(SD))	83.3 (6.7)	85.3 (5.1)	85.8 (3.8)	86 (2.8)	0.072
Education (y(SD))	15.6 (3.3)	15.22 (3.1)	15.9 (2.4)	14.7 (2.4)	0.82
MMSE (SD)	28.3 (1.5)	26.9 (2.1)	19.2 (6)	22.7 (4.5)	<0.0001
***APOE* ε4 status (-/+)**	95-/17+ (15%+)	29-/12+ (29%+)	8-/5+ (38%+)	5-/1+ (17%+)	0.084
***BDNF* genotype (valval/met carrier)**	77/35	29/12	7/6	4/2	0.81

Note: [1]*p*-values are two-sided and derived from Fisher's exact test (for sex, *APOE* ε4 status, and *BDNF* genotype) and either two-sample t-tests (in expression dataset for age at death, education, MMSE, RIN, and PMI) or ANOVA (in imaging dataset for age at scan, education, and MMSE). ROS/MAP = Religious Orders Study / Memory and Aging Project; CN = cognitively normal; non-AD = non-neuropathologically-confirmed Alzheimer's disease; pathoAD = neuropathologically-confirmed Alzheimer's disease; MMSE = Mini Mental Status Exam score at last visit before death; Val = Val/Val homozygotes; Met = Met allele carriers; F = female; M = male; y = years; SD = standard deviation; R = right; L = left.

Out of a total 1 600 tests (160 SNPs x 10 *SORL1* transcripts), 36 remained significant after correction for multiple testing (threshold of $p<8.33\text{x}10^{-4}$); all 36 modeled the same transcript, SORL1-005 (ENST00000534286), as the outcome (Figure 6-3 shows locus-wide interaction P-

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values for SORL1-005 expression in the whole sample (n=441)). The SNP showing strongest interaction effect was rs12364988 (Wald X^2_1=19.09, p=1.25x10^{-5}, n=441), where the rs12364988^{T} allele reduced likelihood of SORL1-005 expression in the *BDNF*^{Val} homozygotes ($OR_{TT:CC}$=0.38, $C.I._{95\%}$=[0.18,0.80]), but greatly increased likelihood of expression among *BDNF*^{Met} carriers ($OR_{TT:CC}$=7.03, $C.I._{95\%}$=[2.42,20.46]) (see Figure 6-4A). Rs12364988, within the 5' region of *SORL1*, was in moderate to strong LD with the remaining 35 SNPs that showed significant interaction with *BDNF* Val66Met in the ROS/MAP sample (D' range=0.80-1, r^2 range=0.35-1, n=441). The region encompassed by these SNPs, stretching approximately 53kb from rs11218301 to rs1784927, includes two haplotype blocks (defined by Gabriel et al., 2002), and, due to LD structure, can be tagged by only three effectively independent SNPs.

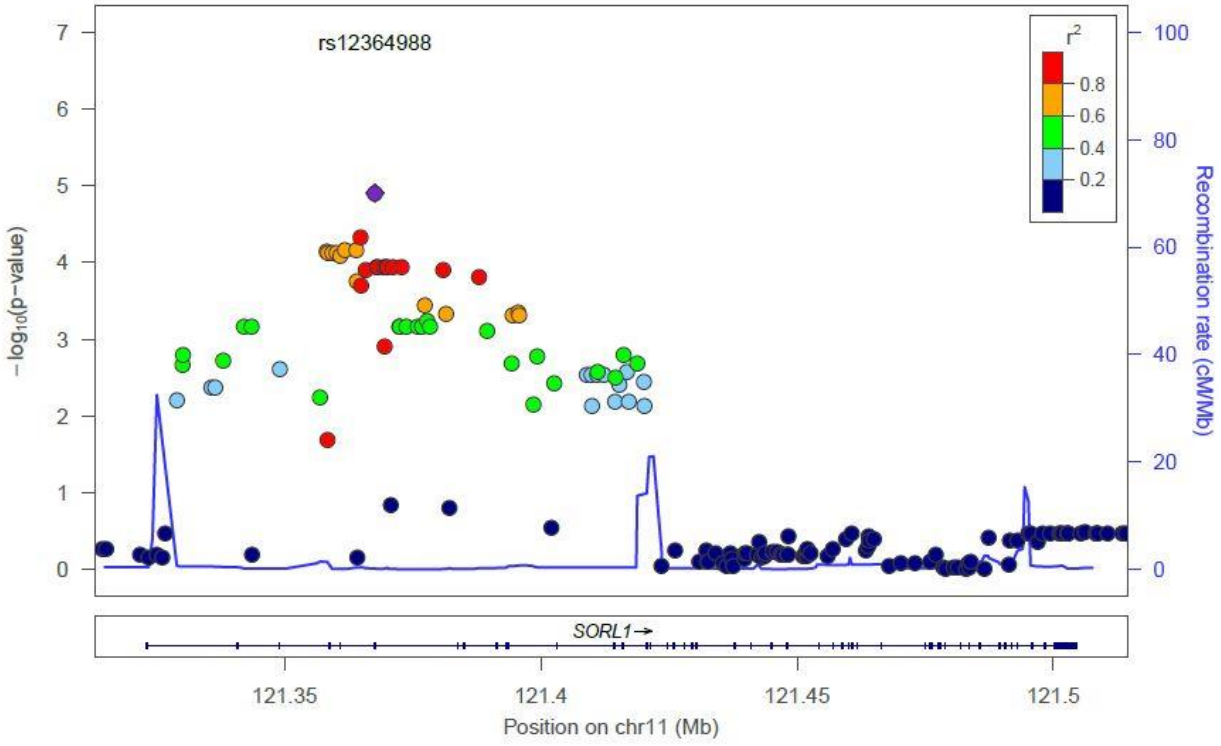

Figure 6-3. –Log(*P*-values) for interaction terms of SNPs across the *SORL1* locus with *BDNF* Val66Met in logistic regression models for expression of SORL1-005 (ENST00000534286). The top interacting SNP was rs12364988 (Wald X^2_1=19.09, p=1.25x10^{-5}, n=441). Colour coding shows LD structure in the region (according to 1000 Genomes hg19 EUR reference), with red indicating high LD (r^2>0.8) and dark blue indicating low LD (r^2<0.2) with respect to rs12364988. Plot was generated using LocusZoom (Pruim et al., 2010).

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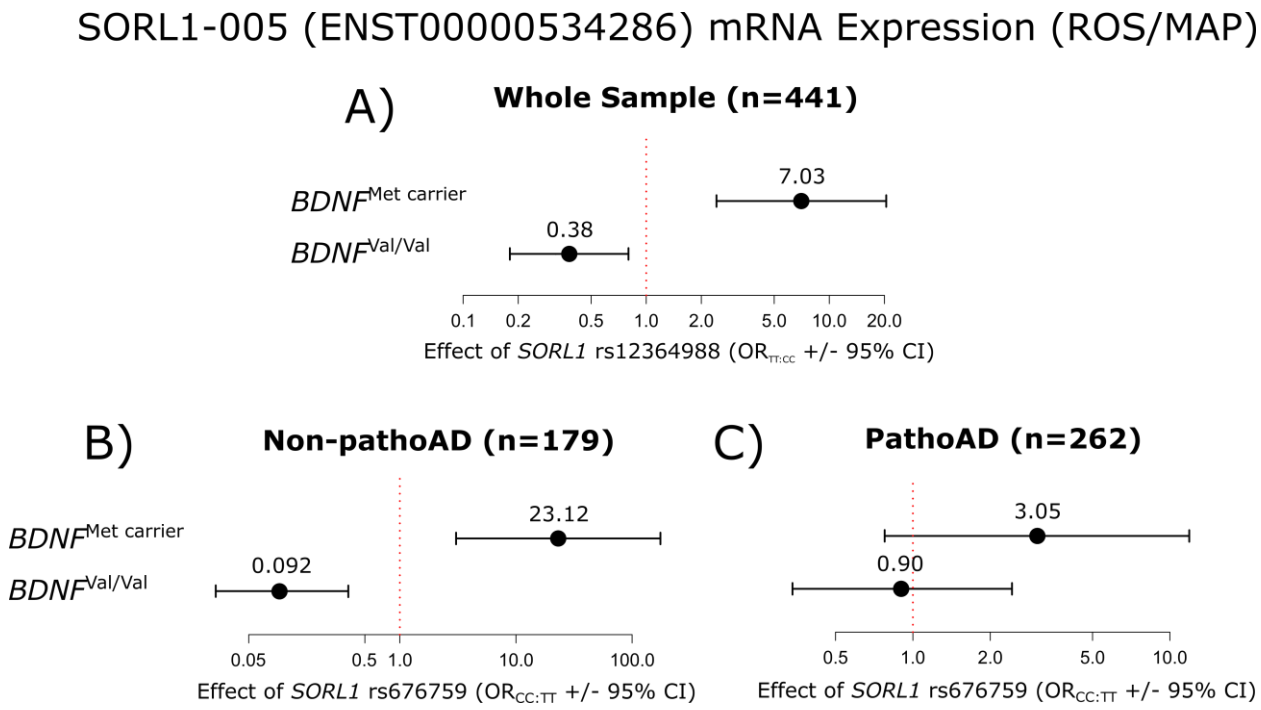

Figure 6-4. Top interaction effects of *SORL1* variants (rs12364988 and rs676759) and *BDNF* Val66Met on prefrontal mRNA expression of SORL1-005 (ENST00000534286) in A) the whole ROS/MAP sample (Wald X^2_1=19.09, p=1.25x10^{-5}, n=441), B) only non-pathologically confirmed AD (non-pathoAD) subjects (Wald X^2_1=19.27, p=1.14x10^{-5}, n=179), and C) pathologically confirmed AD (pathoAD) subjects (Wald X^2_1=1.99, p=0.16, n=262).

Since all interactions showed regulatory effects on the same transcript, SORL1-005, three tests were performed to evaluate the effect of SORL1-005 expression on mid-frontal neuritic plaques, diffuse plaques, total amyloid, PHFtau, and NFTs in ROS/MAP. In the whole sample, when pathology and expression were evaluated as continuous traits, there was an effect of SORL1-005 on diffuse plaques only, whereby increased levels of SORL1-005 were associated with increased number of plaques SORL1-005 ($F_{1,431}$=4.07, p=0.044, n=441). No effects were observed for PHFtau or NFT count (all p_{raw}>0.05). Since both SORL1-005 expression and diffuse plaque pathology levels were heavily skewed (with many subject showing no expression and/or no pathology), a secondary analysis was performed using logistic regression, with SORL1-005 and diffuse plaques both coded as binary variables (*SORL1* expressed vs. not expressed; diffuse plaque pathology above and below third quartile); the effect was preserved (Wald X^2_1=4.42, p=0.036), however, neither result survived correction for multiple testing.

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Post-hoc tests in diagnostic subgroups revealed strongly divergent patterns of effect between non-AD and pathologically-confirmed AD subjects. In the non-AD subgroup (n=179), 44 tests showed interaction *p*-values below the assigned Bonferroni threshold for multiple testing ($P<8.33x10^{-4}$). As in the overall sample, all significant models predicted SORL1-005 expression as outcome, though with much stronger effect sizes observed for the top interacting *SORL1* SNP (rs676759, Wald $X^2_1=19.27$, $p=1.14x10^{-5}$) in the $BDNF^{Val}$ homozygote ($OR_{CC:TT}=0.093$, $C.I._{95\%}=[0.026,0.34]$) and $BDNF^{Met}$ carrier groups ($OR_{CC:TT}=23.12$, $C.I._{95\%}=[3.04,175.36]$) (see Figure 6-4B). Rs12364988 was also among the 44 variants showing significant interaction effects on expression in this subsample (Wald $X^2_1=19.09$, $p=1.24x10^{-5}$). In the pathologically-confirmed AD subset (n=262), no test survived correction for multiple testing (for rs676759, interaction $p=0.16$, see Figure 6-4C), suggesting that the interaction effect is specific to individuals without confirmed AD. In agreement, the effect of SORL1-005 expression on postmortem diffuse plaque neuropathology persisted in the non-AD subjects ($F_{1,169}=3.97$, $p=0.048$), but was absent in pathologically-confirmed AD subjects ($F_{1,253}=2.29$, $p=0.37$).

6.4.2 *In Vivo* Aβ [18F]Florbetapir PET

Sample demographics for ADNI subjects are summarized in Table 6-2. A total of 36 SNP-SNP interactions surviving correction for multiple testing in the expression analyses were analyzed across seven bilateral frontal cortical ROIs for *in vivo* Aβ, resulting in a total of 252 tests. After FDR correction, 44 models remained significant. Between these 44 models, 18 different *SORL1* SNPs demonstrated significant interaction with *BDNF* Val66Met to predict levels of frontal Aβ across five ROIs (out of all significant outcomes (44): lateral orbitofrontal (1), medial orbitofrontal (2), pars opercularis (17), pars orbitalis (7), and pars triangularis (17)). The top SNP showing interaction was rs618874 ($F_{1,649}=12.12$, $p_{raw}=5.3x10^{-4}$, n=710); the $rs618874^T$ allele was associated with decreased amyloid burden in $BDNF^{Val}$ homozygotes, but increased amyloid in $BDNF^{Met}$ carriers (see Figure 6-5). This is in alignment with effects observed on expression and neuropathology in ROS/MAP, whereby the $rs12364988^T$ allele, which is strongly linked to the $rs618874^T$ allele (ADNI $r^2=0.73$; ROS/MAP $r^2=0.74$), resulted in $BDNF^{Met}$-dependent increases in truncated SORL1-005 transcript, which in turn was associated with increased postmortem diffuse amyloid plaques. To test for effects of potential data outliers, we re-

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performed all regressions removing observations lying beyond 1.5 times the interquartile range of mean binding for each ROI. In these analyses, between two and seven subjects were removed from each model; however, statistical significance of our findings were not meaningfully altered. In fact, 52 models remain significant after outlier removal.

Table 6-2. ADNI Sample Demographics

ADNI 1/GO/2 MRI (n=1 285)	**CN (n=335)**	**SMC (n=82)**	**EMCI (n=235)**	**LMCI (n=407)**	**AD (n=226)**	**Diff (*p*)[1]**
Study phase (1, GO, 2)	193 (1), 142 (2)	82 (2)	111 (GO), 124 (2)	291 (1), 116 (2)	131 (1), 95 (2)	<0.0001
Sex (F/M)	159 F, 176 M	51 F, 31 M	101 F, 134 M	155 F, 252 M	102 F, 124 M	0.0012
Age (y(SD))	74.7 (5.4)	71.7 (5.4)	71.0 (7.1)	73.3 (7.4)	74.3 (8.1)	<0.0001
Education (y(SD))	16.2 (2.7)	16.7 (2.6)	16.1 (2.6)	15.8 (2.9)	15.2 (2.9)	<0.0001
MMSE (SD)	29 (1.1)	29 (1.2)	28.4 (1.6)	27.1 (1.8)	23.1 (2.1)	<0.0001
***APOE* ε4 status (-/+)**	251-/84+ (25%+)	53-/29+ (35%+)	132-/103+ (44%+)	175-/232+ (57%+)	68-/158+ (70%+)	<0.0001
***BDNF* genotype (valval/met carrier)**	231/104	54/28	159/76	271/136	154/72	0.96
ADNI GO/2 Amyloid Sample (n=710)	**CN (n=136)**	**SMC (n=88)**	**EMCI (n=244)**	**LMCI (n=121)**	**AD (n=121)**	**Diff (*p*)[1]**
Study phase (GO, 2)	136 (2)	88 (2)	106 (GO), 138 (2)	121 (2)	121 (2)	<0.0001
Sex (F/M)	66 F, 70 M	55 F, 33 M	107 F, 137 M	55 F, 66 M	50 F, 71 M	0.024
Age (y(SD))	73.9 (5.9)	72.2 (5.8)	71.4 (7.4)	72.5 (7.5)	74.3 (8.5)	0.001
Education (y(SD))	16.5 (2.5)	16.8 (2.6)	16 (2.6)	16.5 (2.6)	15.8 (2.6)	0.016
MMSE (SD)	29.1 (1.2)	29 (1.3)	28.3 (1.6)	27.7 (1.8)	23.1 (2.1)	<0.0001
***APOE* ε4 status (-/+)**	104-/32+ (24%+)	60-/28+ (32%+)	130-/114+ (47%+)	51-/70+ (58%+)	43-/78+ (64%+)	<0.0001
***BDNF* genotype (valval/met carrier)**	95/41	58/30	167/77	89/32	79/42	0.66

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Note: *p-values are two-sided and derived from Fisher's exact test (for study phase, sex, *APOE* ε4 status, and *BDNF* genotype) and ANOVA (for age, education, and MMSE). ADNI = Alzheimer's Disease Neuroimaging Initiative; CN = cognitively normal; SMC = some memory concern; EMCI; early mild cognitive impairment; LMCI = late mild cognitive impairment; AD = Alzheimer's disease; MMSE = Mini Mental Status Exam score; Val = Val/Val homozygotes; Met = Met allele carriers; F = female; M = male; y = years; SD = standard deviation.

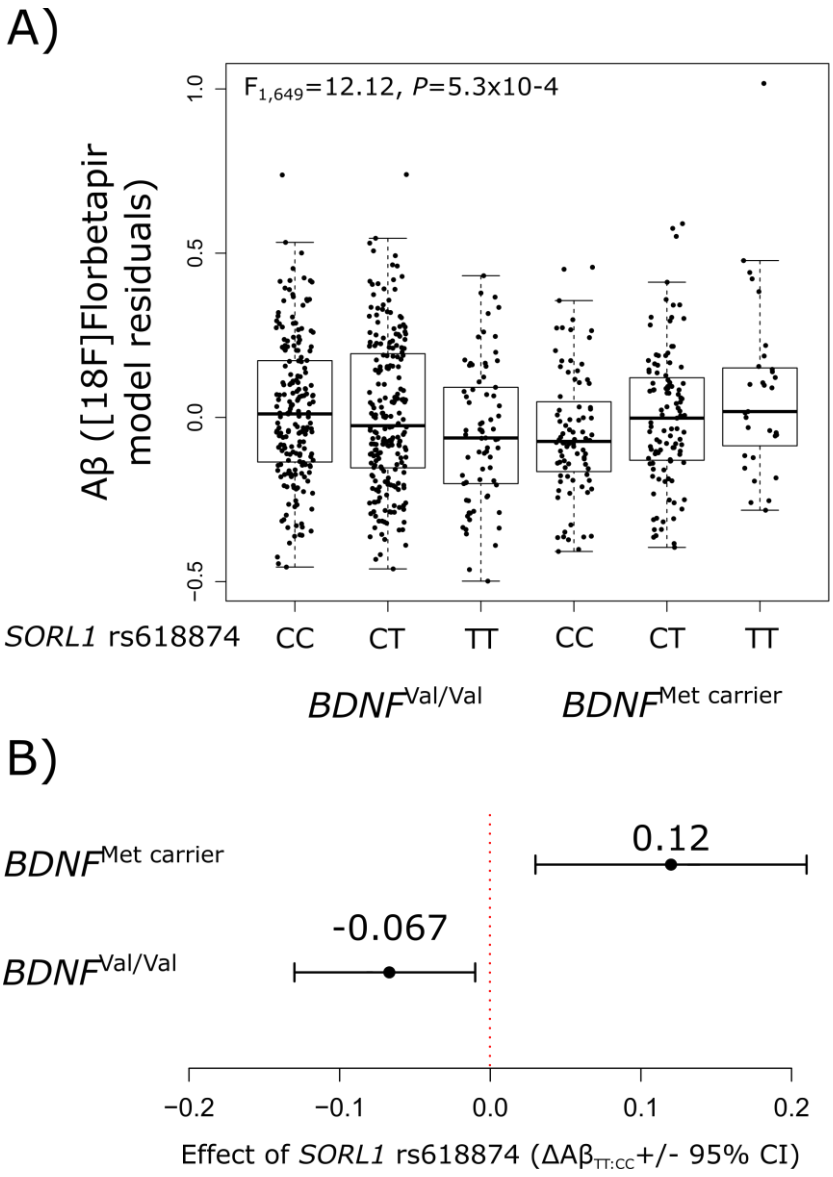

Figure 6-5. *SORL1-BDNF* interaction effect on *in vivo* Aβ in the pars orbitalis measured by [18F]Florbetapir PET in the ADNI 2 sample (n=710). A) Residual amyloid load within genotype-defined groups according to *SORL1* rs618874 and *BDNF* Val66Met. B) Effect of *SORL1* rs618874 within $BDNF^{Val}$ homozygote and $BDNF^{Met}$ carrier groups separately, adjusted for co-variates, with 95% confidence intervals. $Rs618874^{T}$ was associated with decreased amyloid burden in $BDNF^{Val}$ homozygotes ($\Delta A\beta_{TT:CC}$=-0.067, $C.I._{95\%}$= [-0.13,-0.01]), but increased

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amyloid in *BDNF*^{Met} carriers ($\Delta A\beta_{TT:CC}$=0.12, C.I.$_{95\%}$= [0.03,0.21]). Results were not impacted by removal of observations lying beyond 1.5*interquartile range of mean binding.

6.4.3 Entorhinal Cortex Volume and White Matter Microstructure

Exploratory analyses was conducted for each of the 36 significant SNP-SNP interactions from expression analyses on entorhinal cortex volume in both ADNI 1, GO, and 2 (n=1 285) and ROS/MAP (n=172) samples. In ADNI and ROS/MAP, nine and 17 SNPs showed interaction with *BDNF* Val66Met at uncorrected $p<0.05$, respectively; however, no result survived FDR correction for multiple testing. In ADNI, rs662821 showed the strongest interaction with *BDNF* Val66Met ($F_{1,1268}$=4.69, p=0.031), whereby the rs662821^{T} allele was associated with slightly decreased cortical volume in *BDNF*^{Val} homozygotes, but increased volume in *BDNF*^{Met} carriers (see Figure 6-6A). In ROS/MAP, rs12364988 showed the strongest interaction with *BDNF* Val66Met ($F_{1,161}$=6.64 ,p=0.011), whereby the rs12364988^{T} allele conferred a slight decrease in cortical volume in *BDNF*^{Val} homozygotes, but increased volume in *BDNF*^{Met} carriers (see Figure 6-6B). While not significant after FDR correction, these findings were directionally convergent, as in both samples, rs662821^{T} and rs12364988^{T} are strongly linked (ADNI r^2=0.79; ROS/MAP r^2=0.82).

Finally, in 185 subjects from ADNI 2 with DTI data, the interaction of 36 SNP-SNP interactions from expression analyses were tested for effects on FA across five regions bilaterally. At an uncorrected threshold of $P<0.05$, 31 tests were significant and predicted FA of left CBH (1), right IFO (20), left SLF (3), and left SS (7). Rs618874, the top *SORL1* SNP showing interaction effects on *in vivo* Aβ [18F]Florbetapir PET, also showed the most significant interaction with *BDNF* Val66Met on FA ($F_{1,172}$=6.44, p_{raw}=0.012), whereby the rs618874^{T} allele was associated with an average increase in FA of the right IFO in *BDNF*^{Val} homozygotes, but decrease in *BDNF*^{Met} carriers (see Figure 6-6C). No results survived FDR correction.

Given the strong LD structure in the *SORL1* locus, the above findings link higher genotypically-regulated prefrontal SORL1-005 mRNA expression (ROS/MAP) with increased frontal amyloid burden both postmortem (ROS/MAP) and *in vivo* (ADNI). Further exploratory analysis also link this same transcriptional mechanism to greater entorhinal cortex volume (ROS/MAP and ADNI),

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and reduced fronto-temporal-occipital white matter microstructural integrity (ADNI), though these results did not survive correction for multiple testing. All top results for each analyzed phenotype are summarized in Table 6-3.

Table 6-3. Combined Top Results Summary

Phenotype	Sample	N	*SORL1* SNP (hg38 pos)	LD (r^2,D')[a]	Ref allele	*SORL1* Effect Direction		P_{raw}	P_{cor}
						BDNF Met	*BDNF* Val/Val		
SORL1-005 expression	ROS/MAP total	441	Rs12364988 (121496917)	1,1	T	+	-	$1.25x10^{-5}$	$7.5x10^{-4}$
SORL1-005 expression	ROS/MAP non-AD	179	Rs676759 (121488556)	0.63,-0.97	C	+	-	$1.14x10^{-5}$	$1.4x10^{-3}$
SORL1-005 expression	ROS/MAP AD	262	Rs676759 (121488556)	0.63,-0.97	C	+	-	0.16	1
[18F]Florbetapir PET amyloid – pars orbitalis	ADNI 2	710	Rs618874	0.52,-0.93	T	+	-	$5.3x10^{-4}$	0.047
Entorhinal cortex volume	ROS/MAP	172	Rs12364988	1,1	T	+	-	0.031	0.11
Entorhinal cortex volume	ADNI 1, GO and 2	1 285	Rs662821	0.65,-0.99	T	+	-	0.011	0.06
DTI white matter FA - IFOF	ADNI 2	185	Rs618874	0.52,-0.93	T	-	+	0.012	0.39

Note: [a]LD values extracted from HaploReg v4 (Broad Institute, Cambridge, MA) (L. D. Ward & Kellis, 2011). Correction for multiple testing performed according to methods outlined in Statistical Analysis (Section 6.3.3). For expression analyses, Bonferroni correction for 60 tests was performed (6 effectively independent SNPs, 10 *SORL1* transcripts) to identify significant SNP-SNP eQTL interactions. For subsequent analyses of the 36 significant SNP-SNP eQTL interactions identified for SORL1-005, the Benjamini-Hochberg false discovery rate correction procedure was used (Benjamini & Hochberg, 1995). LD = linkage disequilibrium; ROS/MAP = Religious Orders Study / Memory and Aging Project; IFOF = inferior fronto-occipital fasciculus; AD = pathologically confirmed Alzheimer's disease; eQTL = expression quantitative trail loci.

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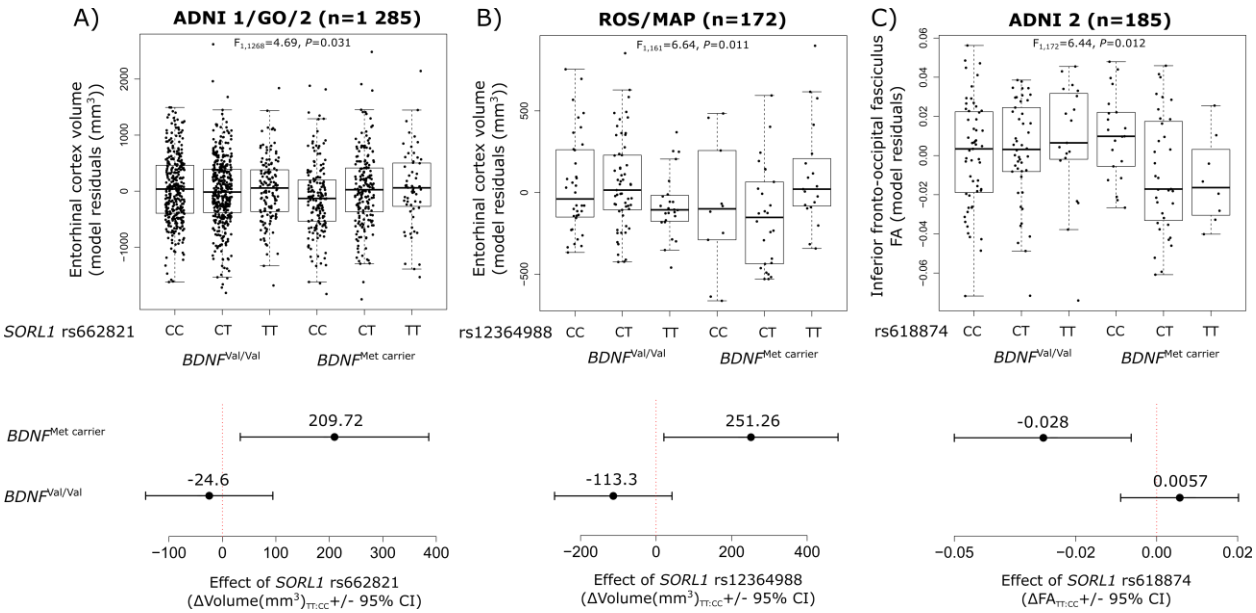

Figure 6-6. *SORL1-BDNF* Interaction Effects on Entorhinal cortex volume (A and B) and white matter FA (C). A) In ADNI 1/GO/2 (n=1 285), the rs662821^{T} allele was associated with slightly decreased cortical volume in *BDNF*^{Val} homozygotes ($\Delta volume(mm^3)_{TT:CC}$=-24.6, C.I.$_{95\%}$= [-143.62,94.36]), but increased volume in *BDNF*^{Met} carriers ($\Delta volume(mm^3)_{TT:CC}$=209.72, C.I.$_{95\%}$= [33.44,386]). B) In the ROS/MAP imaging sample (n=172), rs12364988 showed nominally significant interaction with *BDNF* Val66Met, whereby the rs12364988^{T} allele conferred a nominal decrease in cortical volume in *BDNF*^{Val} homozygotes ($\Delta volume(mm^3)_{TT:CC}$=-113.3, C.I.$_{95\%}$= [-268.82,42.18]), but increased volume in *BDNF*^{Met} carriers ($\Delta volume(mm^3)_{TT:CC}$ =251.26, C.I.$_{95\%}$= [20.7,481.82]). C) In the ADNI 2 DTI dataset (n=185), the rs618874^{T} allele was associated with an average increase in FA of the right IFO in *BDNF*^{Val} homozygotes ($\Delta FA_{TT:CC}$=0.0057, C.I.$_{95\%}$= [-0.0089,0.02]), but decrease in *BDNF*^{Met} carriers ($\Delta FA_{TT:CC}$=-0.028, C.I.$_{95\%}$= [-0.05,-0.0063]).

6.5 Discussion

We found that linked *SORL1* variants within the 5' region of the gene interacted with *BDNF* Val66Met to regulate prefrontal expression of a truncated *SORL1* transcript, SORL1-005 (ENST00000534286), and that this isoform was associated with increased diffuse amyloid plaques in midfrontal tissue from the same subjects. In a second sample, we demonstrated that the same genetic interactions regulating SORL1-005 expression determined frontal amyloid deposition *in vivo* using PET imaging. The gene variant that most significantly interacted with *BDNF* Val66Met to influence SORL1-005 expression, rs12364988, is part of the same highly-linked haplotype block previously shown to interact with BDNF in human iPSC-derived neurons

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to regulate *SORL1* mRNA expression (Young et al., 2015). The three-SNP haplotype block identified in that study was comprised of variants rs668387, rs689021, and rs641120; in our analyses of SORL1-005 expression, these variants each showed significant interactions with *BDNF* Val66Met after correction (all three p=1.2x10^{-4}).

SORL1 is a member of the Vps10p-domain (Vps10p-D) family of neuronal receptors (Willnow, Petersen, & Nykjaer, 2008), several of which have been shown to interact directly with BDNF. For example, SorCS2 has been shown to determine the way in which glia and neurons respond to pro-neurotrophins, including proBDNF, in murine CNS (Glerup et al., 2014). Also, sortilin, another Vps10p receptor, is responsible for intracellular trafficking of newly synthesized proBDNF via its physical interaction with the region of *BDNF* pro-domain in which the Val66Met substitution resides (Z.-Y. Chen et al., 2005). The STRING10 database (http://string-db.org/) shows evidence for indirect interaction of SORL1 with BDNF (score=0.869).

SORL1-005 is a putative protein-coding transcript of 1 124 amino acids (UniProt ID: E9PP43). Based on sequence alignment from the ensembl database, the protein translated from SORL1-005 would lack the Vps10p domain found in the full length protein, potentially interrupting its functions within the amyloid cascade and leading to accumulation of pathogenic Aβ species (Andersen et al., 2005; Caglayan et al., 2014; Rogaeva et al., 2007), consistent with our observations of concomitant increases in diffuse plaque pathology postmortem and amyloid burden *in vivo*. Another potential mechanism via which increased expression of the truncated SORL1-005 transcript may exert pathological effects on brain structure is regulated intramembrane proteolysis (Brown, Ye, Rawson, & Goldstein, 2000); SORL1 has been shown to undergo sequential cleavage by alpha and gamma-secretase enzymes (Nyborg, Ladd, Zwizinski, Lah, & Golde, 2006), liberating protein fragments that are internalized to the nucleus and play roles in gene regulation. It is possible that altered recognition of SORL1-005 by gamma-secretase results in the absence of SORL1 COOH-terminal fragments that may preserve pathologically-protective gene regulation (such absence is also seen in cells co-transfected with FAD-linked *PS1* mutations (Nyborg et al., 2006)). In either case, given SORL1's dual protective roles in recycling APP (Andersen et al., 2005; Rogaeva et al., 2007) and lysosomal targeting of Aβ (Caglayan et al., 2014), alternative splicing causing loss-of-function would be expected to influence amyloid levels in the direction observed in our study. Of note, SORL1-005 has previously been analyzed for association with neuropathology by Yu et al. (Yu, Chibnik, et al.,

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2015) who found no association of SORL1-005 expression with amyloid pathology in the ROS/MAP sample. This discrepancy is due to study parameter differences, as the effect was small and not detected at threshold using permuted *p*-values from multiple tests performed in that study.

The association of SORL1-005 with only diffuse plaques of the midfrontal cortex may suggest a differential contribution of the *SORL1-BDNF* interaction to diffuse vs. neuritic plaque pathology. Diffuse plaques account for the majority of plaque pathology in human brain (Dickson & Vickers, 2001), are associated with AD diagnosis (Yamaguchi et al., 1988), but are also found frequently so-called "normal" aging (Morris et al., 1996), suggesting that they may be indicative of the early, pre-symptomatic stages of disease or even just a non-pathological form of aging. It has been shown that Aβ plays crucial roles in neuroplasticity (Parihar & Brewer, 2010), and may be produced as part of a neuroprotective response to synaptic pathology in AD (H. Lee et al., 2007; Masliah, Terry, Mallory, Alford, & Hansen, 1990); $A\beta_{1\text{-}28}$ has been shown to promote growth and survival of hippocampal neurons (Whitson, Selkoe, & Cotman, 1989). As a result, it is possible that the regulatory action of *SORL1* and *BDNF* may act on amyloid pathways in such a way that influences both neuritic plaque (neurotoxic) and diffuse plaque (neuroprotective) pathologies. Particularly, the interaction effects of variants identified by our study may influence the latter pathway to the greatest degree; hence why genetic effects are only observed in subjects without neuritic plaque and neurofibrillary tangle pathology. Through this lens, BDNF's modulation of resilience via cognitive reserve (D. Ward et al., 2015) is clarified – depending on *SORL1* genotype, the effects of Val66Met may influence protection against AD by promoting diffuse plaque deposition over neuritic. The hypothesis that aging interacts with other factors to render the brain susceptible to AD (Geula et al., 1998) and that amyloid builds as a consequence is supported by our previous observations of *BDNF* Val66Met age-dependent effects on brain structure. [18F]Florbetapir has been shown to accurately measure plaque burden in the brain as indexed by both diffuse and neuritic plaque counts (correlation r=0.95 of [18F]Florbetapir with total plaque score) (Choi et al., 2012), meaning that our findings in the ADNI PET sample (n=710) may be reflective of SORL1-005's effect on diffuse plaques in postmortem frontal cortex. Taken together, we suggest that the interaction between *BDNF* and *SORL1* may provide links between AD risk and the healthy aging process by influencing the expression of a transcript

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that is not related to AD risk, but nonetheless modulates diffuse amyloid deposition postmortem and *in vivo*.

It is also possible that the transcriptional mechanism behind the effect may be subject to either interruption or masking by AD-related neuropathology. It has been shown that Aβ oligomers are capable of inducing gene expression changes across diverse functional classes in human brain tissue (Sebollela et al., 2012), and that genes involved in intracellular trafficking specifically show marked down-regulation in postmortem AD brain (S. A. Small et al., 2005). These findings may have implications for identifying gene regulatory mechanisms in AD brain, as the effects of transcriptional machinery may be altered depending on the level of cellular pathology present.

Previous investigations of the effect of *SORL1* variation on the expression of SORL1 isoforms have shown mixed results, with some studies demonstrating that *SORL1* variants are associated with the preferential expression of SORL1 protein isoforms, but not with levels of *SORL1* mRNA (Caglayan et al., 2012). Others have shown that common variants are associated with total *SORL1* mRNA in temporal cortex, and with exon-skipping specifically in frontal cortex (McCarthy et al., 2012). Others have found no effects of genotype on expression of SORL1 (Dodson et al., 2006). We have previously found that 5' *SORL1* variants influence the expression of total *SORL1* mRNA in an age-dependent manner, whereby genotypic differences were only observed early in life (Felsky et al., 2014). The implications of alternative splicing for the interpretation of SORL1 quantity to AD risk are potentially wide-reaching; differences in isoform expression have been observed between CN elderly and AD patients (Grear et al., 2009), and studies looking at total *SORL1* expression have found increases (Furuya et al., 2012), decreases (Scherzer et al., 2004), and no differences (Sager et al., 2012) between AD and CN subjects.

Our observation that *SORL1* variants interacted with *BDNF* Val66Met to influence entorhinal cortex volume as well as FA of several white matter tracts suggests that the same genetic mechanism determining SORL1-005 expression may also influence brain structure. We chose to analyze entorhinal cortex as it is one of the earliest brain regions to be affected by AD pathological lesions and atrophy (H. Braak, Thal, Ghebremedhin, & Del Tredici, 2011), finding that the genotypically-defined groups showing increased SORL1-005 expression and amyloid pathology also showed increases in entorhinal cortical volume in two independent samples. In

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agreement, it was recently shown that entorhinal cortex volume is actually increased in healthy individuals genetically at-risk for AD (DiBattista, Stevens, Rebeck, & Green, 2014), though this study included much younger subjects (average age ~22y) than were analyzed here. A reason why individuals with greater amyloid pathology might also have increased entorhinal volumes lies in *BDNF* Val66Met's role in moderating access to cognitive reserve (D. Ward et al., 2015). The interaction of *BDNF* with *SORL1* may have parallel neuroprotective effects on entorhinal structure as a compensatory mechanism against increasing pathology. For effects on white matter microstructure, the directionality of effect aligns with the majority of work on microstructural alterations of white matter in AD, with consistent reductions in FA observed for MCI and AD subjects (H. Huang et al., 2012; J.-H. Wang et al., 2013). Our top association with inferior fronto-occipital fasciculus is not surprising; it connects to the temporal lobe, is a late-myelinating fiber, and is associated with AD risk (Stricker et al., 2009; Voineskos et al., 2011), consistent with the retrogenesis model of AD development and progression. It should be emphasized, however, that no interaction effects on brain structure in this study surpassed our experimental threshold for multiple correction, despite directional congruence between samples for each phenotype tested.

BDNF Val66Met has been associated with risk for AD (Fehér, Juhász, Rimanóczy, Kálmán, & Janka, 2009) and AD-related intermediate phenotypes (Y. Y. Lim et al., 2013; Voineskos et al., 2011), albeit inconsistently,(Ji et al., 2015) and is thought to be an important factor in modulating neuroplasciticty (Mizui et al., 2015; Ninan et al., 2010). Our results may provide insight into the conflicting literature surrounding the effect of *BDNF* Val66Met (i.e. why it has not been identified by GWAS for AD diagnosis or pathology); the vast majority of studies in this area have not accounted for *SORL1* genotype and thus may be missing crucial information determining the direction and magnitude of *BDNF*'s effects on disease risk. The mechanisms via which *BDNF* Val66Met influences downstream risk for AD are complex and not yet understood; recently it was shown that *BDNF* Val66Met alters the expression of miR-146 in humanized *BDNF* knock-in mice (Hsu, Xu, Mukai, Karayiorgou, & Gogos, 2015), suggesting that this variant may influence the expression of multiple target genes simultaneously.

The present study has several limitations. First, the decision to analyze gene expression as a binary outcome necessarily introduces a level of bias into the analyses; it is possible that by splitting the distributions of transcript expression into expressed vs. not expressed, we missed

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quantitative information that here we would have been unable to test without violating statistical assumptions. Second, the expression of *SORL1* has been shown to be cell-type specific (Scherzer et al., 2004), whereby some individuals with AD have loss of expression n neurons, but not glia. We are unable to test this directly in our sample, as the ROS/MAP expression data are derived from tissue homogenate of the prefrontal cortex. Therefore, it is possible that noise due to cell non-specificity played some role in our results; though it is unlikely that this would generate false positives given that cell-specific changes in *SORL1* expression would more likely serve to dilute signal within a mixed-cell population. Also, we took steps to maintain regional specificity in our analyses by analyzing frontal pathology, which should help mitigate some concerns over differences between regions. Third, as with any RNA sequencing experiment, alignment error must be considered as a potential confounder. Finally, BDNF's effects on amyloid pathology (Rohe et al., 2009; Young et al., 2015) as well as TrkB-dependeng trophic signaling (Rohe et al., 2013) have been shown to depend on SORL1, and in this study we used the functional Val66Met variant as an indirect proxy for brain BDNF activity (Egan et al., 2003). However, there is inconsistency in the literature surrounding the influence of Val66Met on BDNF protein and mRNA expression in blood and brain tissue that highlights the uncertainty of this assumption. In postmortem brain, Val66Met has been shown to influence cerebellar *BDNF* mRNA expression (Burgess et al., 2015). In blood, it has been shown that $BDNF^{Val}$ homozygotes show lower levels of BDNF than $BDNF^{Met}$ carriers in Generalized Anxiety disorder (GAD) (Moreira et al., 2015), while other studies and meta-analyses of disease populations and healthy subjects have found no effect of *BDNF* Val66Met genotype on plasma or serum BDNF (Jamal, Van der Does, Elzinga, Molendijk, & Penninx, 2015; Kreinin et al., 2015; Luykx et al., 2013; Suriyaprom, Tungtrongchitr, & Thawnasom, 2014; Terracciano et al., 2013; Y. Wang et al., 2015).

In conclusion, we have demonstrated a gene-gene interaction between two AD risk-associated genes that impacts the isoform-specific expression of *SORL1*, amyloid deposition, and potentially brain structure, in two large samples. We believe that this interaction may provide insight into the convergence of prototypical neurotoxic Aβ deposition and the brain reserve found in aged individuals who are resilient to AD pathology. This work has implications for the way that genetic association studies of *SORL1* and *BDNF* are interpreted and may be of use in determining specific groups of genetically at-risk individuals in future clinical trials of novel therapies directed toward amyloidogenic and neuroplastic mechanisms.

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Chapter 7

7 General Discussion and Future Directions

7.1 Overview of Findings

The goal of this work was to identify the phenotypic impact of functional genetic variants that operate within convergent AD risk pathways and in doing so, shed light on the roles of those pathways in AD (genes and gene variants depicted in Figures 7-1 and 7-2).

In the first study (Chapter 3), we demonstrated that the major AD risk factor *APOE* ε4 was associated with microstructural qualities of the cingulum bundle in an age-dependent manner, providing a structural substrate for observed inefficiencies in hippocampal engagement during memory recall as measured with fMRI. This contribution provides an explanation for discrepancies of *APOE*'s effect in the literature and suggests links between vascular changes associated with ε4 status may manifest differently at different ages. The deficits in white matter microstructural integrity observed in late life ε4 carriers are consistent with the literature on AD risk (Section 1.3.7). The early life effects of *APOE* ε4 and the early changes in FA measurable in healthy subjects who go on to develop a-MCI (Zhuang et al., 2012) both point toward mechanisms acting at the early stage of illness. As outlined in Section 1.2.3, amyloidogenesis is one such early-acting mechanism, and this led us toward the next gene candidate, *SORL1*, which codes for an APOE receptor and regulates Aβ production.

In the second study (Chapter 4), we examined the effects of well-established *SORL1* gene variants on white matter microstructure in two independent samples, but went further to identify mechanisms of action by analyzing two postmortem samples for gene expression and neuropathology. We found that *SORL1* risk variants were associated with consistent decreases in FA across the lifespan, as well as early loss of *SORL1* expression and late-life accumulation of Aβ. In contrast to our study of *APOE*, the age-independent effects of *SORL1* suggest an "early hit' mechanism, whereby an early life event (perhaps change in gene expression) elicits changes in white matter that are preserved and do not accelerate with age. As described in Sections 1.2.3 and 1.2.6, recent ammendments and revisions of the amyloid cascade hypothesis have brought the accumulation of Aβ neuropathology and neuroinflammation side-by-side; the so-called

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"amyloid cascade-inflammatory hypothesis" (McGeer & McGeer, 2013). This inexorable link between amyloid and inflammatory pathways led us to the next gene candidate, *TSPO*, which codes for a receptor that may modulate the response of microglia to SORL1-dependent Aβ accumulation.

In the third study (Chapter 5), we probed *in vivo* and postmortem the effect of a functional variant within the *TSPO* gene, which is thought to be a marker for neuroinflammation, by comprehensively analyzing neuroinflammatory and cerebrovascular phenotypes in three separate cohorts. We examined *in vivo* cerebral infarcts, white matter hyperintensities and plasma inflammatory biomarkers, as well as postmortem infarcts, cerebral amyloid angiopathy and microglial activation. Against expectation, we found no replicable effect of the *TSPO* variant on any phenotype of interest. Following our in-depth characterization of these three genes (*APOE*, *SORL1*, and *TSPO*) independently, and building on prior work from our group characterizing the AD-associated *BDNF* Val66Met mutation (Voineskos et al., 2011), we next sought to develop a more complete picture of the genetic effects we and others had previously observed by specifically analyzing gene-gene interaction effects (Section 1.4.8).

In the fourth and final study of this thesis (Chapter 6), based on evidence from recent human stem cell experiments, we tested the interaction effects of *SORL1* and *BDNF* genotypes on *SORL1* mRNA isoform expression and white matter FA in two independent samples. This was the first examination of genetic effects on *SORL1* expression using RNA-sequencing, and we were able to demonstrate a significant interaction between *SORL1* and *BDNF* that aligned with *in vivo* experiments showing *SORL1* genotype-dependent effects of BDNF administration on SORL1 expression.

Together, these findings suggest that common genetic variants in *APOE* and *SORL1* contribute independently to the changes in AD risk biomarkers in healthy subjects and, in the case of *SORL1*, may parse some of the heterogeneity of amyloid levels in subjects with MCI and AD. This work also demonstrates that binding properties of an important neuroinflammatory receptor, TSPO, are not reflected by alterations in brain vascular lesions, blood-based inflammatory biomarker levels, or the activation of microglia, and that interactions between variants of *SORL1* and *BDNF*, proposed as a major contributor to the "missing heritability" of AD (Zuk et al., 2012), may have large effects on basic cellular mechanisms that contribute to transcriptomic

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diversity and ultimately altered neuropathological profiles. Using the information in this thesis, it may be possible to delineate molecular subtypes of subjects with genotypes that render them susceptible to particular neuropathological pathways or deficient for potentially protective factors. These insights were made possible by the powerful combination of genetics and neuroimaging, complimented by next-generation RNA sequencing and unique postmortem brain samples with detailed neuropathological assessments.

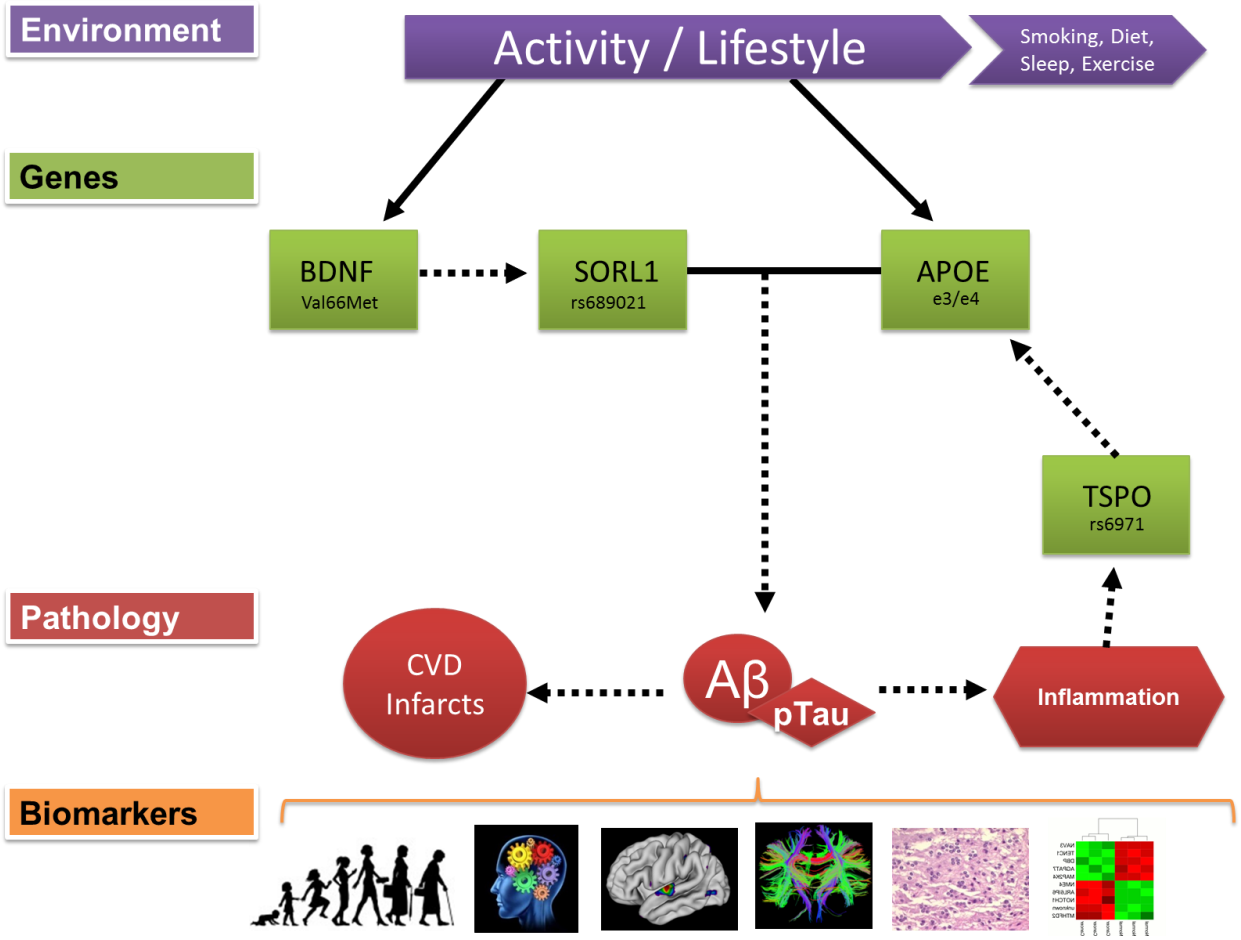

Figure 7-1. Summary of related genes analyzed in this thesis, their connections to eachother, and a basic diagrammatical representation of how genes mediate the effects of environment on pathology found in AD. Pictoral depictions of AD biomarkers on the lower quadrant from left to right: aging, cognition, cortical morphology, white matter tract microstructure, measurements of neuropathology, and gene expression array.

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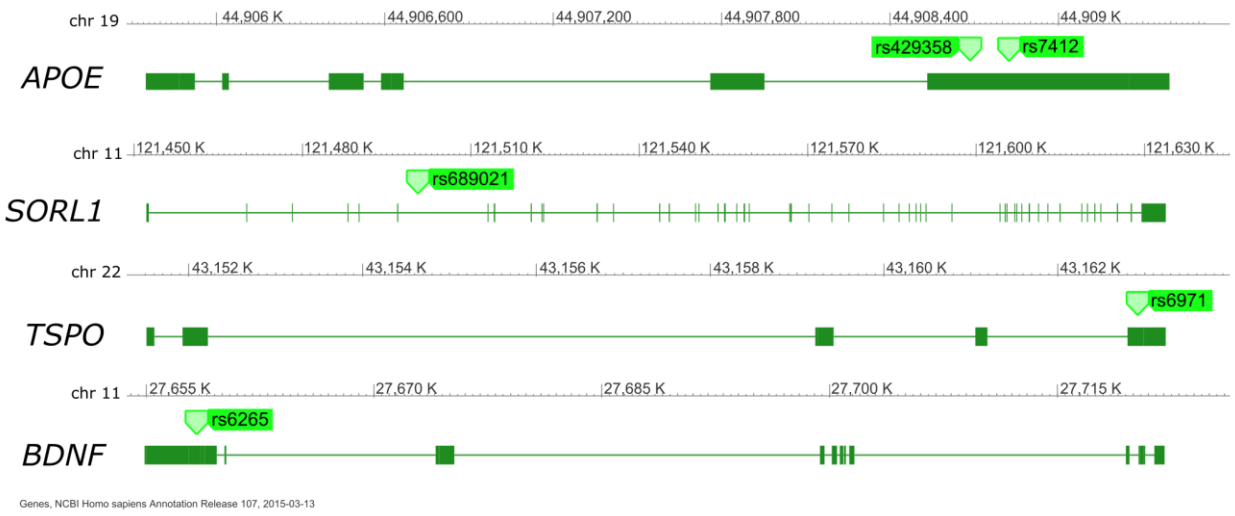

Figure 7-2. Contextual positions of gene variants analyzed in each study for *APOE*, *SORL1*, *TSPO*, and *BDNF*.

7.2 Limitations

There are several important limitations to the approaches presented in this thesis that should be considered when drawing conclusions from this work. Some are specific to the study designs employed, while others are more general but nonetheless deserve mention. First, all data presented in this thesis is from cross-sectional analyses. The overarching question of risk for disease can only be answered definitively by prospective, longitudinal study designs. Therefore, many assumptions have been made regarding the interpretation of the preceding data, especially in the context of predicting future outcomes and supposing rates of change. We acknowledge that these study designs cannot infer causality, but rather draw associations between variables of interest that may represent upstream, concomitant, or downstream processes. It should also be noted, however, that the nature of genetic studies is that in nearly all cases (at least where effects of somatic mutation can be reasonable ruled out), the determination of genotype is known to have occurred before the development of the phenotype being measured. This provides a reliable timeline and lends some legitimacy to the inference of causality regarding genetic associations.

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Second, the totality of genetic variation within genes included in this thesis was not examined. SNPs were chosen based on extensive *a piori* data suggesting their functional relevance to AD and effects on related biomarkers (outlined in Section 1.4). While this candidate approach is well-nested in the existing literature, inter-study variability and uncertainty surrounding estimates of risk effects or proposed functions means that there is always room for further discovery. Gene variants other than those investigated in *APOE* and *BDNF* have been implicated in disease, and it is possible that the gene variants included in our studies are effectively tagging genetic variation at unobserved causal loci. For the specific variants investigated in our study, this may not be as such of a concern, since there is a substantial bodies of literature characterizing the functional consequences of *APOE* ε4, *BDNF* Val66Met, and *TSPO* rs6971. For *SORL1*, the direct consequences of gene variation are not as well established, however, we addressed this in the final study, where interactions with *BDNF* were considered at all *SORL1* variants locus-wide, not just at those previously associated with AD. This approach, and the use of polygenic risk scores that capture gene variation within and across loci, are discussed below as future work.

Third, several samples of cognitively normal elderly subjects were analyzed in the present studies. While the goal is ultimately to apply our findings to the general population, these samples, which would contain some individuals with non-penetrant brain pathology, may represent atypically resilient groups of people. Beyond the aforementioned resilient qualities in the ROS sample (Negash et al., 2011), the lack of penetrance of AD pathology is an inevitable source of sampling bias, as healthy control lifespan samples in our studies are recruited based on a lack of clinical symptoms and not the absence of underlying neuropathology. Young participants may go on to develop dementia in late life, whereas recruited elderly are known to be free of cognitive deficits (either due to lack of pathology or resilience to it). This means that simple, linear conclusions about the relationships between genes, observed brain structure and pathology, and risk for AD may not accurately reflect the underlying processes. For example, in Chapter 4, the *SORL1* alleles that have been implicated in risk for AD (Rogaeva et al., 2007) were those associated with increases in white matter tract FA and early life *SORL1* mRNA expression, as well as less late life accumulation of amyloid. Despite this drawback, the discrepancy between allelic association with brain biomarker data and AD diagnosis is not uncommon (Bralten et al., 2011; Cuenco et al., 2008), and in fact this directional "inconsistency"

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may very well represent resilient compensatory networks in individuals who are susceptible to AD via insult to other brain regions or systems (discussed in Section 7.4.1).

Finally, a number of technical and biological limitations in MRI studies must be considered. Important technical confounds include motion artefacts (changes in MR signal due to the movement of subjects in the scanner can impact volume and thickness estimates (Reuter et al., 2015)), magnetic field distortions and inhomogeneities (Styner, Brechbuhler, Szckely, & Gerig, 2000; D. Wang, Strugnell, Cowin, Doddrell, & Slaughter, 2004), intrinsic scanner properties (Fu, Fonov, Pike, Evans, & Collins, 2006) (inter-site variability may hinder cross-experiment replication and combined analyses without careful consideration (Thompson et al., 2014)), and computer software differences (Pipitone et al., 2014) (different image processing methodologies can have drastic consequences for output values). Other confounds that are much less often corrected for include biological contributors to the variance in MR signal, such as time of day (Hastings, Reddy, & Maywood, 2003), menstruation (Hagemann et al., 2011), dehydration (Duning et al., 2005), physical activity (C. J. Smith et al., 2014), and inflammation (Braskie et al., 2014). Like with structural MRI, but perhaps to a greater extent, technical and biological confounds are often major concerns with DWI. For example, heart pulsation may cause local changes in brain parenchyma volume (Poncelet, Wedeen, Weisskoff, & Cohen, 1992) and impact DWI signal (Skare & Andersson, 2001). For more detail on limitations of DWI and DTI, and the strategies used to address them, see Tournier et al. (Tournier, Mori, & Leemans, 2011). Also, the use of single-shot echo-planar imaging sequences for DWI renders the acquisition especially susceptible to motion and other B0 field-induced artefacts; though some of these concerns have been mitigated by parallel imaging and multi-shot acquisition sequences (Pruessmann, Weiger, Scheidegger, & Boesiger, 1999; Soares, Marques, Alves, & Sousa, 2013).

7.3 Immediate Questions Raised

Each study in this thesis raises a number of questions that can be used moving forward to generate hypotheses and guide further research. Chapters 3 and 4 adopt lifespan approaches to draw some conclusions about the effect of genetic variation at different points in the lifespan. This is important because, despite AD being a late-life disease, the pathogenesis of AD is

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arguably underway as early as childhood (Growdon & Hyman, 2014; Trommsdorff et al., 1999). In postmortem brain tissue from 42 young individuals aged 4-29, Braak et al. (H. Braak & Tredici, 2010) found that all showed early changes in tau proteins. In a much larger follow-up study of 2 332 brains including those from subjects as young as one year of age, Braak et al. (H. Braak et al., 2011) again found abnormal tau across the age range. However, the presence of even the earliest stages of AD pathology may not be sufficient to state that someone is on the path to AD. It was recently suggested that the presence of NFTs and absence of neuritic plaques may characterize a condition termed "primary age-related tauopathy" (PART), rather than a pre-symptomatic form of AD (Crary, 2014). Braak and Del Tradici (H. Braak & Del Tredici, 2015) criticize this theory, however, citing a lack of postmortem evidence for a tauopathy showing only ghost NFTs in the absence of other early tauopathic lesions. Despite the evidence for a more direct association of NFTs with cognition than Aβ, it has been shown that NFTs may reside in host neurons for several decades without destroying them (kordower 2001), suggesting that manifestation of neurogenerative process may be conditional upon other age-related factors. In children and adolescents, *APOE* ε4 carrier status has been associated with volume reductions and cortical thinning in the temporal lobe (La Joie, Crowley, Wendelken, Bunge, & Jagust, 2014), specifically left entorhinal cortex thickness shows a stepwise relationship to *APOE* status, where ε4< ε3< ε2 (Shaw et al., 2007). Even infant ε4 carriers show alterations in brain structure, with less myelination and lower gray matter volume than ε4 non-carriers in the cingulate, lateral temporal, and occipitotemporal regions, and greater myelination and volume in frontal regions (Dean et al., 2014).

Most studies of AD risk genes and brain structure have been in elderly populations. Those studies examining genetic effects across the human lifespan are few (especially for genes other than the ubiquitous *APOE*), and the work on *SORL1* in this thesis represents some of the first efforts of this type. Interpreting the effects of genetic variants on AD biomarkers at different stages of the lifespan (or between healthy and dementia populations) therefore is a tricky business and, as emphasized in this thesis, should be approached cautiously. We demonstrated that *APOE* shows an age-dependent effect on cingulum bundle white matter microstructure. This could be interpreted in multiple ways. Potentially, 1) the function of APOE4 protein within biochemical pathways over the course of life as age-related physiological changes occur, or 2) early etiopathological changes arising from consistent APOE4 dysfunction spur developmental

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compensatory processes, however, these effects erode quickly with time (faster than in neutral ε3 homozygotes), ultimately resulting in late-life white matter alterations and AD risk compared to ε3. Interestingly, a recent report published after our *APOE* study found that the effect of *APOE* on cognition may be mediated by white matter tract integrity (Lyall et al., 2014). In contrast, we observed age-independent effects of SORL1 on white matter integrity in the same sample as well as a second replication sample. This could also be interpreted in multiple ways: 1) *SORL1* variants in the 5' region impact the development but not the rate of decline of white matter, possible due to inaction in later life (i.e. loss of genetic effect on gene expression), or 2) 5' *SORL1* variants impact pathogenic processes both early and late in life, but decline in white matter in old age due to deleterious alleles is successfully counteracted by compensatory pathways, potentially related to *BDNF*. Both of these possible interpretations are supported by our follow-up work in the BrainCloud postmortem sample (Section 4.4.2), finding early life changes in SORL1 gene expression due to genotype and no difference in elderly. Other potential mechanisms and experiments to assess them are discussed in Section 7.4.3.

The question of age-dependent and –independent effects of genetic variants is important in the larger clinical picture of AD and its early stages. While a-MCI is a high-risk state for progression to AD, not all subjects with a diagnosis of a-MCI will develop AD; MCI is much more unstable than AD. This could be due to more ambiguous diagnostic criteria, or the fact that at this stage in the disease process, multiple brain pathways are adapting to aging as well as any additional pathological insults (analogous to the way the body responds to infection), a process that may be more successful in some than others (see resilience, Section 7.4.1). In clinical trials of neuroactive treatments, such as transcranial magnetic stimulation (TMS) or transcranial direct current stimulation (tDCS), which targets specific brain circuitry, the temporal effect of AD risk genes may be of critical importance. For example, CAMH has recently been awarded a $10 million, five-year grant (Preventing Alzheimer's dementia with Cognitive remediation plus tDCS in MCI and Depression (PACt-MD)) from Brain Canada and the Chagnon Family to test the efficacy of tDCS in preventing dementia in subjects with MCI or depression. Brain imaging will be used to assess the efficacy of this treatment. However, if genetic information and its age-associated pattern of effect on the brain are not taking into account, results may be confounded. This confounding has been demonstrated in studies of neuroinflammation, where the primary outcome measure, TSPO binding, is heavily dependent on the rs6971 polymorphism (Owen et

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al., 2012), and administration of pharmacological agents targeting this outcome has strong genotype-dependent effects (Owen et al., 2014). In fact, the NIH now requires that funded clinical trials demonstrate engagement of the therapeutic target (i.e. brain structure, function, or pathology), and so genetic variants with effects on these measures (potentially discrepant at different stages of life and disease) should be accounted for.

7.4 Future Directions and Next Steps

7.4.1 Cognitive/Brain Reserve and Resilience

Perhaps counter-intuitively, it has been suggested that the factors contributing to the risk for AD are not the same as those contributing to the pathology that is associated with AD (Chui et al., 2012). Part of this notion comes from the idea that "pure" AD pathology underlies most cases of dementia, when in fact this is increasingly being recognized as a rare phenomenon (J. A. Schneider et al., 2007). A large proportion of subjects with no signs of dementia are found to have significant AD-related neuropathology at autopsy. This gap between cognitive performance and neuropathology is termed "reserve" (Stern, 2002, 2012). Two models of reserve are 1) brain reserve and 2) cognitive reserve. According to the brain reserve model, an individual with greater brain reserve capacity (e.g. larger brain, more neurons etc.) would be more resilient to a given amount of neurological insult (Katzman et al., 1988). In contrast, cognitive reserve takes into account the dynamic nature of neural networks and describes the brain's ability to sustain normal operation despite damage to its neural substrate (Stern, 2002), potentially through more efficient utilization of existing brain circuitry. Epidemiological observations support both brain reserve and cognitive reserve models, and these concepts are widely used to explain the gap between neuropathology and the degree of cognitive impairment (Dufouil, Alpérovitch, & Tzourio, 2003; Glatt et al., 1996; Schofield, Logroscino, Andrews, Albert, & Stern, 1997; Stern et al., 1994).

Another explanation for the apparent gap between neuropathologic alterations and cognitive impairment may be unobserved or unknown pathologies. The likelihood of clinical dementia increases with multiple coexisting pathologies (J. A. Schneider et al., 2007). Thus, prior studies only accounting for limited pathologies (e.g. amyloid and tau) would show an apparent discrepancy between pathology and cognition (as in the Nun study (Snowdon et al., 1997), where

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the presence of infarcts explained apparent outliers in the pathology-cognition relationship). Furthermore, details not captured by conventional neuropathologic assessment (such as amyloid plaque size, subtypes of oligomeric amyloid, and synaptic tau multimers) may also explain some of the mismatches between pathology and cognitive outcome (Perez-Nievas et al., 2013).

The concepts of brain and cognitive reserve are thematically present throughout the literature, even if not explicitly stated. For example, it has been shown that aerobic exercise over 6-months in elderly is associated with increased volume of both gray and white matter (Colcombe et al., 2006), a classic explanation for brain reserve due to physical activity. The relationship between AD genes and resilience is an increasingly popular area of study. It has been shown that physical activity is able to protect against hippocampal volume reduction over 18 months, but only in *APOE* ε4 carriers (C. J. Smith et al., 2014). One promising approach to studying cognitive resilience involves the calculation of a "residual cognitive score", which is a single number generated for each individual in a study that represents the deviation from a level of cognition that is expected based on their level of brain pathology. As a proof of concept, a GWAS in 750 subjects from ADNI with "residual cognition" (after controlling for age, sex, education, and pathologies measured by structural MRI (stroke (Hachinski score), infarcts, hippocampal volume, cortical volume, and WMH volume)) found that a variant within the *RNASE13* was genome-wide significant (Mukherjee et al., 2012). This top variant was in high LD with SNPs mapping to the *TPPP2* gene - a member of the α-synuclein family of proteins - indicating that perhaps the residual cognitive score was associated with unobserved Lewy body pathology. **Future examinations of a "residual cognitive score" could shed light on the processes underlying resilience to pathology, either by identifying truly resilient functions or illuminating important unobserved pathological features in AD and aging.**

7.4.2 Locus-Wide Analyses, Polygenic Scores, and DNA Sequencing

The concern that common genetic variants currently studied in GWAS and other candidate approaches do not capture the real underlying, causal gene variants for disease or disease biomarkers is substantiated. It has been estimated that only between 24-33% of phenotypic variation in AD is explained by known common variants (S. H. Lee et al., 2013; Ridge et al., 2013), whereas AD is ~80% heritable (Gatz et al., 2006). While this "missing heritability" and

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the potential role of interaction has been discussed and addressed in Chapter 6, other hypotheses states that gene variants that are not genotyped by available assays or observed in a given study are those responsible for disease, and that the statistical signal observed in current genetic association studies are diluted proxies of these causal variants. Also, there are many other types of common genetic variation, such as copy number variants (CNVs) and microsatellites that are often completely ignored, often due to technological or costs restraints. An example of a gene in which types of sequence variation other than SNPs may influence important AD phenotypes is the translocase of outer mitochondrial membrane 40 homolog (*TOMM40*) gene, which lies within an LD block encompassing *APOE* and *APOC1*. Allen Roses, the discoverer of the *APOE* association with AD, identified a poly-T structural variant (rs10524523) that significantly predicted the age-at-onset of AD in *APOE* ε3 homozygotes (Roses et al., 2010), effectively refining and building upon the association of that genomic locus with AD phenotypes. **Future studies of the genes analyzed in this thesis should consider these additional sources of genetic variability and, as implemented in Chapter 6, apply locus-wide analyses to reduce noise due to LD and better localize the causal variation.**

Among recent developments in AD genetics is the use of polygenic risk scores to evaluate cumulative genetic contributions to AD phenotypes (Desikan et al., 2015). Polygenic risk scores are single values calculated for an individual based on sum of risk alleles for any number of a priori selected genetic variants, which may be weighted by their effects on disease status or some other phenotype of interest. Work in AD has shown that polygenic risk scores derived from top GWAS-significant SNPs (not including *APOE* ε4) are associated with AD risk (Marden, Walter, Tchetgen Tchetgen, Kawachi, & Glymour, 2014) and AD-related biomarkers such as CSF $A\beta_{42}$ and cortical thickness (Sabuncu et al., 2012) in independent samples. This lends credence to the GWAS method as useful for discovery of potentially meaningful AD-related variants. However, recent analyses from the Cognitive Ageing Genetics England and Scotland (CAGES) consortium in over 3 000 cognitively normal elderly found that the AD polygenic risk score was not associated with baseline or change in cognitive ability (S. E. Harris et al., 2014), suggesting that genetic contributors to AD are distinct from those associated with cognitive decline in **. Future work would benefit from the development of polygenic risk scores that are specific to gene expression or resilience biomarkers in aging and AD.**

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The common disease, rare variant hypothesis stipulates that rare mutations with high penetrance are at the root of disease risk and that common SNPs may tag these variations via LD structure (N. J. Schork et al., 2009). DNA sequencing allows for the detection of sequence variation at any site, not just those pre-determined in a chip-based genotyping assay. However, major limitations to this method currently include increased cost compared to genome-wide genotyping and the need for very large sample sizes to detect and statistically analyze rare variants. This method has yielded consistent results (though publication bias should always be considered) implicating rare variation in a multitude of complex disorders and phenotypes, including type I diabetes (Nejentsev, Walker, Riches, Egholm, & Todd, 2009), obesity (Ahituv et al., 2007), heart disease and cholesterol (Cohen et al., 2006), and colorectal cancer (Azzopardi et al., 2008). Of particular relevance, rare coding variation in *SORL1* has been found in individuals with early onset AD (Pottier et al., 2012). Initiatives to sequence the genome in large groups of AD and control subjects, such as the NIH-funded Alzheimer's Disease Sequencing Project (ADSP) (Childress et al., 2014) make analyses of rare variation in candidate genes feasible without significant investment. **Future analyses should include the contributions of both common and rare genetic variation.**

7.4.3 Verification of Underlying Mechanisms

There are many biological mechanisms by which a genetic variant may exert downstream phenotypes. In Chapter 6, we examined the consequences of a regulatory *SORL1-BDNF* interaction on alternative splicing, or the production of mRNA transcript isoforms from the same genetic sequence. Sequence-dependent modification of trans- and cis-acting regulatory mechanisms is an active field of investigation. The Encyclopedia of DNA Elements (ENCODE) project was launched in 2003 (The ENCODE Project Consortium, 2012) is ongoing collaboration aiming to functionally characterize genetic variation across the human genome, including effects on transcription factor binding and the function of remote long-range regulatory elements. ENCODE has played a major role in dispelling dogma surrounding so-called "junk DNA", which was the colloquial term originally ascribed to non-coding sequences of DNA (~98% of the genome), by demonstrating the ~80% of the human genome has biochemical function. This information is readily available on commonly used bioinformatics portals such the

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University of California, Santa Cruz (UCSC) Genome Browser. Efforts led by the Allen Institute for Brain Science (Seattle, WA) have developed a three-dimensional map of gene expression and anatomy in the human and mouse brain, allowing for *in silico* analyses of regional gene expressions for candidate genes (Sunkin et al., 2012). Using the Allen Brain Atlas, one can easily determine gene co-expression profiles based not only on magnitude of expression but also on topographical similarities, an important consideration when seeking explanations for region-specific effects of genetic variants. Another dataset, called the Genotype-Tissue Expression (GTEx) project has been made publicly available by the Broad Institute of MIT and Harvard (Cambridge, MA), and allows for the quick integrated analyses of genetic effects on gene transcript levels across tissue types, including different brain regions (Lonsdale et al., 2013). Pilot data from this project includes an average of 28 tissue samples from 237 postmortem donors, on which DNA genotyping (at 4.3 million variants) and RNA sequencing (at a depth of 82.1 million mapped reads per sample) (The GTEx Consortium, 2015). **Future studies of genetic variation would be wise to use *in silico* analyses in these and other publicly available resources to determine putative genetic function to guide choices of follow-up genes for laborious and costly *in vitro* experimental manipulation.**

Mechanisms of gene regulation not due directly to sequence variation are known as epigenetic modifications, which themselves may be influenced both by genetic variation and environmental factors. Epigenetics and gene expression mechanisms are at the foundation of the brains response to injury and are altered in AD (Lord & Cruchaga, 2014); mouse models of AD utilize both gene mutation and gene overexpression constructs to elicit their disease phenotype (Webster et al., 2014). Epigenetic factors influence gene expression by controlling the accessibility of DNA sequence to the cell's transcriptional machinery; chromatin, the DNA/protein complex found in cell nuclei, can be either open/active (euchromatic) or closed/inactive (heterochromatic). Established indicators of chromatin state, and therefore transcriptional activity, are histone protein modifications (e.g. acetylation of H3K9) and DNA CpG sequence methylation, whereby acetylation of the H3K9 histone mark indicates active transcription (Z. Wang et al., 2008) and CpG methylation indicates the blocking of transcription (P. A. Jones & Takai, 2001). Combining genetic sequence data with epigenetic data and gene expression data derived from next-generation RNA sequencing may make it possible to dissect, at multiple levels, how genetic variation functions independently or dependently of epigenetic phenomena. Since a gene that is

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not expressed cannot influence downstream phenotypes, the modeling of genetics in combination with epigenetics and gene expression can also help inform which genes are likely causal.

Evolutionary information may also be used to gain insight into the mechanisms underlying genetic effects on disease risk. Some have suggested that the ε4 allele persists evolutionarily due to balancing selection (the so-called Charlesworth-Martin hypothesis), as evidence shows it is protective against liver damage in hepatitis C infection (Finch & Morgan, 2007). Interestingly, the estimated prevalence of *APOE* ε4 relates linearly to latitude in a manner that is ethnicity-dependent: frequency declines with increasing northern latitude in Africans (R^2=0.32) and Asians (R^2=0.16), but increases in Europeans (R^2=0.30) and North Americans (R^2=0.57) (P. P. Singh et al., 2006). This also has implications for studies of genetic effects in different ethnic groups, as the frequency of risk-associated alleles may differ greatly between and even within populations in such a way that relates to other environmental, potentiating factors. For example, Africans living closer to the equator may have a higher prevalence of *APOE* ε4, however, they also have more sunlight exposure and consequently lower risk for vitamin D deficiency (Gilchrest, 2008), which is linked to AD (Annweiler, Llewellyn, & Beauchet, 2013). **Further work evaluating genetic risk factors within ethnic groups (including Caucasians) should consider population substructure factors such as geographical location.**

7.4.4 Evaluation of Potential Clinical Utility

There remains an important unanswered question; what constitutes clinical utility? How much research and what level of confidence is required to use genetic information for diagnostic and prognostic decisions? These are hotly debated questions, and with respect to genetic testing in AD, their answers are not clear (Atkins & Panegyres, 2011; J. P. Evans, Skrzynia, & Burke, 2001). A Pubmed search for articles including APOE (or apolipoprotein E) and aging, dementia, or Alzheimer's in their titles or abstracts alone (search terms: ((APOE[Title/Abstract]) OR (apolipoprotein e[Title/Abstract])) AND (aging[Title/Abstract] OR dementia[Title/Abstract] OR alzheimer[Title/Abstract] OR alzheimer's[Title/Abstract])) OR Apolipoprotein E4) currently yields 6 809 studies, compared to similar searches for other AD risk genes and their proteins (*PSEN1*=2 344, *BDNF*=1 124, *PSEN2*=672, *SORL1* = 153, *TSPO*=95) (retrieved Sept 18, 2015). And yet, information on *APOE* ε4 status, the most widely replicated genetic AD risk factor, was

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deemed "not sufficiently specific" to warrant a diagnosis of probable AD "with increased level of certainty", in the 2011 NIA-AA diagnostic criteria (McKhann et al., 2011). Despite decades of work and thousands of studies, no genetic tests currently exist for the detection or prediction of AD. While it is not logical to believe that perfectly discriminative or predictive tests can or should be developed from genetic information alone (as even the highest heritability estimates suggest that environment accounts for ~20% of variance in AD (Gatz et al., 2006)), **the determination of how to use genetic data smartly in the clinic, perhaps incorporating gene-gene and gene-environment interactions into predictions, should be a focus of future research**. For example, some evidence suggests that *SORL1* variants other than those analyzed in this thesis may interact with sex to elicit effects on hippocampal atrophy and whole brain volume (Assareh et al., 2014).

In order to assess the authenticity of genetic associations with disease biomarkers, the quality of outcome phenotypes is of the utmost importance. The term "endophenotype" (or intermediate phenotype) was first coined by Bernard John and Keith R. Lewis (John & Lewis, 1966) and introduced to the field of psychiatry by Irving Gottesman and James Shields (Gottesman & Shields, 1973). An endophenotype is a heritable biomarker that co-segregates with the illness in question, is state independent, and is found in family members without the disease (Gottesman & Gould, 2003). These criteria have been amended several times (including that the phenotype be part of the causal process of disease, or that it manifest at certain points in the lifespan) (Flint & Munafò, 2007), but the essential idea remains the same: endophenotypes are less complex than the diseases they represent, hence they are easier to dissect and more representative of the underlying disease process. The biomarkers examined in this thesis all meet criteria for endophenotype and thus represent meaningful, AD-related phenomena with genetic underpinnings. However, the literature (as reviewed in Chapter 1) remains heterogeneous surrounding the effects of many AD-implicated genes on established endophenotypes, both within and between diagnoses (Reitz & Mayeux, 2009). Work to enhance the resolution, specificity, and sensitivity of currently available endophenotypes is already underway; a recent study from our group has demonstrated that a novel technique known as neurite orientation dispersion and density imaging (NODDI) (H. Zhang, Schneider, Wheeler-Kingshott, & Alexander, 2012) that uses diffusion MRI to estimate neuritic density and organization in gray matter, may be a valid *in vivo* representation of the cortical dendritic disruption that is commonly

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found in aging (Dickstein, Weaver, Luebke, & Hof, 2013). The NODDI-derived orientation-dispersion index (ODI) is able to predict chronological age with greater accuracy than cortical thickness or white matter FA in the same group of subjects (Nazeri et al., 2015). In order to go further, our understanding of the heterogeneity of AD and the relative roles of different etiopathological mechanisms is required – the work in this thesis may shed light on the phenotypic presentation of pre-symptomatic AD subjects with genetic risk backgrounds.

The use of genetic information in risk assessment for AD, where truly causal gene variants are unknown, is a contentious area (Karlawish & Green, 2014). Communicating results of such an assessment to the lay population is difficult, time consuming, and may incite undue panic. Still, companies such as 23andMe, Navigenics, and DeCODEme are offering genetic testing and instant delivery of results direct-to-consumer in the general population. While some basic protections afforded by the Genetic Information Nondiscrimination Act (GINA) (Hudson, Holohan, & Collins, 2008), including those related to insurance and employee privacy, have been established in the United States, no such legislation has been enacted in Canada (Walker, 2014). Furthermore, the heterogeneity in the literature is reflected in the inconsistency of disease risk estimates between services (Kalf et al., 2014). Optimistically for the future of personalized risk assessments and ultimately personalized medicine, it seems that disclosure of well-established risk genotypes (such as *APOE* ε4) does not result in clinically meaningful post-disclosure distress (R. C. Green et al., 2009). A noninferiority trial has shown that condensed disclosure protocols (e.g. those performed briefly in clinic by a general practitioner, as opposed to at length by a trained genetic counsellor) result in no higher incidence of anxiety or depression following disclosure of *APOE* genotype (R. C. Green et al., 2014). Intriguingly, however, Lineweaver et al. (Lineweaver, Bondi, Galasko, & Salmon, 2014) have shown that the awareness of *APOE* ε4 carrier status is enough to affect objective cognitive performance in healthy older adults. Those *APOE* ε4 carriers who were aware of their status performed worse on verbal memory tasks than those ε4 carriers who were unaware. Further, it has been shown that knowledge of the absence of *APOE* ε4 does not effectively alleviate concern over perceived risk (Chilibeck, Lock, & Sehdev, 2011). This raises important questions about the way that the concept of disease "risk" is interpreted, and demonstrates the need for informative and actionable gene-biomarker associations.

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7.5 Final Conclusions

An editorial published during the final stages of preparation of this thesis (Au, Piers, & Lancashire, 2015) made recommendations to the field that the focus of AD research be on identifying molecular subtypes of the disorder for specific targeting of novel therapies. The work presented here attempts to do this and signals that progress is being made. For example, therapies impacting early life development of white matter, particularly association fibres connecting to the temporal lobe, may be most effective in those homozygous for 5' *SORL1* risk variants. Currently, our lab is conducting trials of TMS (albeit in individuals with schizophrenia) to test its effects on brain structure, including white matter.

In Sum, AD is primarily a genetic disorder, and both independent and interactive effects of specific genetic variants can be measured on brain-related biomarkers of AD risk and progression. These effects may be age-dependent or age-independent, and implicate multiple etiopathogenic pathways related to cerebrovascular disease, Aβ accumulation, neuroinflammation, and neurodegeneration, reflecting the heterogeneous composition of AD risk. Further studies should focus on encompassing all sources of genetic variation, consider the interactive and cumulative effects of these variants, account for epigenetic modifications, and refine outcome phenotypes to maximize statistical power and potential clinical utility of findings. If successful, future work as well at that presented in this thesis may facilitate the identification of genetically at-risk molecular subtypes and development of novel treatments aimed at AD onset delay or prevention.

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