Genetic markers of neurodegeneration: a role for paraoxonase 1 in sporadic Alzheimer's disease?

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There is only one good,
knowledge,
and one evil,
ignorance.

Socrates

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Abstract

Mounting evidence points to increased oxidative stress and disruption of cholesterol homeostasis as being crucial events implicated in Alzheimer's disease (AD) etiopathology. Paraoxonase1 (PON1) is a high-density lipoprotein-associated antioxidant enzyme that functions to protect both lipids and the cholinergic system, the primary neurotransmitter system affected in AD, from respectively oxidation and organophosphate compounds. Using a large and homogeneous cohort of Quebec citizens, we therefore sought to investigate the involvement of PON1 in AD. We found that a single nucleotide polymorphism (SNP) in the PON1 gene not only exerts an AD risk enhancing effect but also a paradoxical survival rate increasing effect and a later age at AD symptom onset. This SNP is also significantly associated, sometimes in opposite directions for both sexes, with beta-amyloid levels, senile plaque accumulation and choline acetyltransferase activity in numerous brain areas. Thus, these results suggest a prominent role for PON1 in the etiopathology of AD.

Résumé

Maintes preuves confirment l'implication d'une augmentation du stress oxydatif et d'une perturbation de l'homéostasie du système cholestéro-lipidique dans le développement de la maladie d'Alzheimer (MA). La paraoxonase 1 (PON1), une enzyme antioxydante liée aux lipoprotéines de haute densité, est reconnue pour sa capacité de protéger tant les lipides que le système cholinergique, l'un des premiers systèmes de neurotransmetteurs affectés par la MA, contre respectivement l'oxydation et les composés organophosphorés. En utilisant un large groupe de citoyens québécois, nous démontrons qu'un polymorphisme génétique de la PON1 augmente le risque de développer la MA tout en repoussant paradoxalement l'âge de mort et de début de la maladie. Ce polymorphisme affecte, de façon parfois opposée chez les deux sexes, les niveaux de protéine bêta-amyloïde, de plaques séniles et d'activité de l'acétylcholine transférase dans plusieurs régions du cerveau. Nos résultats suggèrent donc un rôle prépondérant de la PON1 dans le développement de la MA.

Acknowledgments

Research is a fountain of knowledge just waiting to be tapped. What better place to drink deeply from it than McGill University, one of the world's leading university and undisputed leader in the teaching of neurological sciences? Few areas of research are as complex as the studies of neurodegenerative diseases, and my utmost gratitude therefore goes to my supervisor, Dr Judes Poirier, who shared with me a lot of his expertise, research insight, critical thinking and fighting spirit. You quickly became for me the role model of a successful researcher in the field. Thank you for your guidance, your wise advice, and for trusting and believing in me.

I'm grateful for the opportunity to have studied and pursued my research project in a state of the art research institute such as the Douglas Hospital Research Center, where science can be learned at its best. I feel very lucky to have been a part of the stimulating and competent research team of Dr Poirier's Alzheimer's disease Research Laboratory. I wish to thank all the past and present members of the lab, notably Stephanie Bélanger Jasmin, Louise Théroux, Doris Dea, Nicole Aumont, Vanessa Pearson and Véronique Legault, for their help and advice. Stephanie, thank you for your emotional and professional support, your helpful suggestions and for standing beside me as a caring colleague. Louise, I feel indebted to you for your never fading optimism and endless smiles, as well as for always having a 5minute time to spare, listen and help me. Doris, the rigor and devotion you put to your work are essential researcher qualities to which I will always draw.

I wish to thank the Canadian Institute of Health Research (CIHR) for the partial studentship that made this work possible. I would also like to thank the members of my advisor committee, Drs Josephine Nalbantoglu and Heather Durham, for their time and effort spent on this project.

Finally, and most importantly, I dedicate this work to my loving family: my parents, my grandmother, my brothers and their wives, my uncles, aunts and cousins, and my loving boyfriend, Jilbert, for their enthusiasm and support. Jilbert, thank you for your wise advice, immediate support and love I have relied upon during my time at the university. Dad, mom, your faith in me and encouragement is what kept me going and the reason why this thesis has seen the light of day. I have succeeded because of you.

Merci pour tout. Je vous aime profondément.

Contributions of authors and non-authors

The work presented in this thesis is the fruits of labor of many graduate students and research assistants. I would like to express my gratitude to all those who contributed to make this thesis possible.

Two manuscripts have been written in relation to the work presented here, one has been published at the beginning of 2008, and the other, which is presented in chapter 3, was to be submitted to the *Human Molecular Genetics* journal at the moment of this thesis submission.

Paper 1: Leduc, V., and Poirier, J. (2008) Polymorphisms at the paraoxonase1 L55M and Q192R loci affect the pathophysiology of Alzheimer's disease: emphasis on the cholinergic system and beta-amyloid levels. *Neurodegener. dis.*, **5(3-4)**, 225-227. (1)

Paper 2: Leduc. V., Théroux, L., Dea, D., Robitaille, Y., and Poirier, J. Involvement of paraoxonase 1 genetic variants in Alzheimer's disease etiopathology *(Submitted to Human Molecular Genetics)*.

Contributions of authors

Valérie Leduc, MSc candidate

I conducted the vast majority of work (90%) pertaining to paraoxonases' (PONs') genotyping assays, namely the design of primers, the optimization of experimental conditions for both polymerase chain reaction (PCR) and restriction enzyme digestion, as well as the PCR amplification and the digestion of PCR products for eleven single nucleotide polymorphisms. I also performed the entire real time reverse transcriptase-PCR (RT-PCR) analysis, notably the design of primers, the determination of the single best out of 10 candidate reference genes for the normalization of expression data, and the mRNA quantification of PON1 and PON2 in the frontal cortex of 90 Alzheimer's disease and non-demented

control subjects. Even though these analyses are only partially completed, I wish to mention that I performed the immunoblot analysis (protein quantification) of PON1 and PON2, and I developed a kinetic assay to quantify PON1 arylesterase activity in post-mortem brain tissues. Finally, I performed all the statistical analyses presented in this thesis.

Louise Théroux, BSc

She participated in the PON1 genotyping effort (10%), and collected and classified all brain tissue samples that were used in this study (from the Douglas Hospital Brain Bank).

Doris Dea, MSc

She conducted the entire apolipoprotein E genotyping, and extracted DNA from brain tissues (for use in genotyping experiments).

Yves Robitaille, MD

He performed all neuropathological examinations, including senile plaque and neurofibrillary tangle counts.

Judes Poirier, PhD

He is the research project's initiator. He also provided precious constructive comments on the study and technical designs throughout the project, and participated in the composition of the manuscripts.

Contributions of non-authors

Uwe Beffert, PhD

He performed the entire beta-amyloid peptide quantification in brain tissues.

Isabelle Aubert, PhD

She assessed, in collaboration with Danielle Cécyre, the choline acetyltransferase activity in brain tissues.

Danielle Cécyre, MSc

She assessed, in collaboration with Isabelle Aubert, the choline acetyltransferase activity in brain tissues. As the coordinator of the Douglas Hospital Brain Bank, she also made human brain tissues available for us to use in this study.

Véronique Legault, MSc

She extracted the vast majority of RNA (85%) from brain tissue samples (for use in real time RT-PCR experiments). Valerie Leduc extracted the RNA from the remaining brain tissue samples (15%).

Contributions to knowledge

The following experimental observations represent original and useful contributions to knowledge:

1. Paraoxonase 1 L55M and Q192R genetic variants affect total beta-amyloid 40 and 42 peptide levels, as well as beta-amyloid 42/40 ratio in the post-mortem frontal cortex and hippocampus tissues of non-demented control and Alzheimer's disease subjects.

2. Paraoxonase 1 L55M genetic variants affect neuritic senile plaque accumulation, in opposite direction for men and women, in multiple human post-mortem brain areas of Alzheimer's disease subjects.

3. Paraoxonase 1 L55M genetic variants affect the choline acetyltransferase activity in multiple human post-mortem brain areas of non-demented control and Alzheimer's disease subjects.

4. Paraoxonase 2 A148G and C311S genetic variants affect the choline acetyltransferase activity in the hippocampus of non-demented control and Alzheimer's disease subjects.

5. Paraoxonase 1 L55M genetic variants affect the mRNA expression of paraoxonase 1 in the post-mortem frontal cortex tissue of apoE4^{+} non-demented control subjects.

6. Relative to non-demented control subjects, paraoxonase 1 and 2 mRNA levels are upregulated in the post-mortem frontal cortex tissues of Alzheimer's disease subjects.

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List of abbreviations

Acetylcholine (ACh)
Acetylcholinesterase (AChE)
Alzheimer's disease (AD)
Amyloid precursor protein (APP)
Anterior cingulate cortex (ACC)
Apolipoprotein (Apo)
ApoE2 receptor (ApoE2R)
Arginine (Arg, R)

Brodmann area (BA)

Beta-amyloid (Aβ)

Bovine serum albumine (BSA)

Calcium (Ca2+)

Central nervous system (CNS)

Cerebrospinal fluid (CSF)

Choline acetyltransferase (ChAT)

Cholinesterase inhibitor (ChEI)

Cornus Ammonis (CA)

Dentate gyrus (DG)

Early-onset AD (EOAD)

Endogenous reference gene (ERG)

Familial AD (FAD)

Glutamine (Glu, Q)

Hardy-Weinberg equilibrium (HWE)

Hazard ratio (Exp(B))

High-density lipoprotein (HDL)

Histidine (His, H)

Horizontal diagonal band of Broca (Hdb)

3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR)

4-Hydroxynonenal (HNE)

Kilobase (Kb)

Late-onset AD (LOAD)

LDL receptor (LDLR)

Leucine (Leu, L)

Low-density lipoprotein (LDL)

Medial septal nucleus (MS)

Megabase (Mb)

Methionine (Met, M)

Multivariate analysis of variance (MANOVA)

National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRADA)

Neurofibrillary tangle (NFT)

Nucleus basalis of Meynert (NB)

Odds ratio (OR)

Organophosphatase (OPase)

Organophosphate (OP)

oxidized HDL (oxHDL)

oxidized LDL (oxLDL)

Paraoxonase1 (PON1)

Peripheral nervous system (PNS)

Phenylmethyl sulfonyl fluoride (PMSF)

Phosphatidylcholine (PC)

Phosphatidylethanolamine N-methyltransferase (PMET)

Phospholipase-A2 (PLA2)

Polymerase chain reaction (PCR)

Post-mortem delay (PMD)

Presenilin (PS)

Pyruvate dehydrogenase kinase 4 (PDK4)

Quebec French Canadian (QFC)

Reverse transcriptase polymerase chain reaction (RT-PCR)

Senile plaque (SP)

Single nucleotide polymorphism (SNP)

Sporadic AD (SAD)

Vertical diagonal band of Broca (Vdb)

Ubiquitin-conjugate enzyme E2D2 (UBE2D2)

Ideas in the mind are the transcript of the world;
words are the transcript of ideas;
and writing and printing are the transcript of words.

Joseph Addison

CHAPTER 1 – INTRODUCTION

Research rationale

Specific objectives

Thesis organization

Chapter 1. Introduction

1.1 – Research rationale and specific objectives

Genetic studies have identified several genes that are involved in causing or predisposing carriers to Alzheimer's disease (AD), a progressive neurodegenerative brain disorder. For the familial sub-type of the disease, which accounts for less than 2% of all cases, AD can be elicited by mutations on genes localized on chromosomes 1, 14 and 21 (2, 3). While no dominant genetic mutation has been identified, numerous prevalent genetic risk factors have been uncovered for the common form of the disease (also refer to as sporadic AD). These include, but are not limited to, apolipoprotein E (apoE) 4 allele on chromosome 19 (4, 5), alpha$_2$-macroglobulin on chromosome 12 (6), lipoprotein lipase on chromosome 8 (7, 8), and two apoE receptors called LRP (9, 10) and SORL1 (11) on chromosomes 12 and 11, respectively. Interestingly, all of the preceding markers were shown to play a very active role in the maintenance of phospholipids and cholesterol homeostasis in the central nervous system (CNS).

As such, could phospholipids and cholesterol play a pivotal role in AD etiology? The answer is most probably yes. First, cholesterol is not only a fundamental component of cell membranes and myelin sheets, it also appears to be the limiting factor of synaptogenesis *in vivo* (12, 13), rate of which is reduced in AD. Second, while cholesterol, as the precursor to all steroid hormones including glucocorticoids (14), may contribute to regulate inflammation, a primary event in the course of AD, phosphatidylcholine (PC) may provide the choline precursor for the synthesis of acetylcholine (ACh) (15), the primary neurotransmitter system affected in AD (16, 17). Third, lipids are crucially involved in synaptic remodeling and plasticity (12, 13), and these key processes occur during learning, formation of memories and neuronal repair, all of which are impaired in AD (12, 13).

Against this background, we choose to use association studies – which have been proposed as useful tools allowing the filtering of large sets of candidate genes in an attempt to identify those potentially playing a functional role in an illness onset and progression (18)– to address whether paraoxonase 1 (PON1) is implicated in AD etiology. Among various other molecules, we choose PON1, a high-density lipoprotein (HDL)-associated antioxidant enzyme. Indeed, given its recognized role in protecting HDL and low-density lipoprotein (LDL) particles against oxidative modifications in the periphery (19-24), PON1 appears as a pivotal candidate for the maintenance of cholesterol and phospholipids homeostasis in the CNS. Numerous genetic studies have investigated the possible implication of PON1 genetic variants in AD, but the issue remains controversial as some studies demonstrate an association (25-33), whereas others failed to replicate this finding (34-38). Herein, we sought to explore and clarify the possible existence of genetic associations between PON1 genetic variants and AD on the risk of developing the disease, on its age of onset, and on its overall duration. To strengthen our findings, we also aimed to search for possible effect of PON1 genetic variants on core pathological hallmarks of AD, namely beta-amyloid (Aβ) peptide levels, senile plaque (SP) and neurofibrillary tangle (NFT) accumulation, and choline acetyltransferase (ChAT) activity.

1.2 – Thesis organization

PON1 has been extensively studied in the fields of cardiovascular diseases and atherosclerosis, and the vast majority of studies pertaining to the study of PON1 therefore used human serum or plasma as tissue samples. It was in this context that I was given the opportunity to assess PON1 mRNA expression, protein and activity levels in human post-mortem brain tissues. Given the poor quality of our tissues relative to fresh plasma/serum tissues, our studies required extensive optimization, and only the PON1 mRNA expression levels were successfully determined within the time constraints imposed by McGill University.

Because the mRNA levels cannot be corroborated by PON1 protein or activity levels, the results presented in this thesis will focus on the genetic analyses we performed, which are presented in chapter 3. The mRNA levels and data pertaining to PON1 activity levels, which are presented in appendices, will only be shortly discussed in chapter 4.

As such, the thesis is organized as follows. In chapter 2, we provide some background discussion on AD, brain lipid metabolism and PONs. The main results of our genetic studies are then presented in chapter 3. A general discussion of the thesis is presented in chapter 4, along with additional results not presented in chapter 3. Possible future extensions of this research, based on our mRNA levels, are also briefly exposed in this same chapter. Finally, a general summary of the results and an overall conclusion are presented in chapter 5.

Let us watch well our beginnings,
and results will manage themselves.

Alexander Clark

CHAPTER 2 – LITERATURE REVIEW

Chapter 2. Literature review

2.1 – Alzheimer's disease (AD)

2.1.1 – Clinical symptoms

Dementias are progressive brain disorders characterized by a decline in cognitive functions beyond what might be expected from normal aging. Of these dementias, AD is the most common type among the elderly, accounting for about 60% of all dementia cases diagnosed (2, 3). The clinical symptoms begin with mild forgetfulness regarding recent events, activities or names of familiar persons and objects. These symptoms advance to memory lapses interfering with daily activities, such as brushing teeth or eating with a spoon; increased difficulty in sorting out names and faces of family and friends; impaired communication skills; and major personality changes. Finally, the symptoms further worsen and result in a near complete absence of communication skills, and a loss of control of bodily functions leading to the need for full-time care and eventually to death (2).

Clinical variations such as differences in age at onset and rate of progression are common among affected individuals, and complicate the correct diagnosis of AD (2, 39). However, the development of criteria-based approaches to the diagnosis of AD, such as the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) Work Group criteria, have considerably improved the clinical diagnostic accuracy, which rate is now around 85-90 per cent (2). Nevertheless, the observation of SPs, and to a lesser extent NFTs, at autopsy is still required for a definite diagnosis of AD (2).

2.1.2 – Neuropathological features

At the macroscopic level, AD is characterized by multiple neurochemical and neuropathological lesions such as SPs and NFTs, which are non-exclusive disease markers since they are found in a multitude of other disorders (2, 39). While not individually unique to AD, these markers have a characteristic distribution and density in the disease [see section 2.1.2.2] (2, 39). The two most famous pathological features of AD are the extracellular SPs, primarily composed

of Aβ peptides, and the intracellular NFTs, primarily resulting from the hyperphosphorylation of the microtubule-stabilizing Tau proteins (fig.2.1-a) (reviewed in 39). Neuronal and synaptic loss, an altered cholinergic system function, and the presence of inflammation, of oxidative lesions, and of numerous acute-phase reactants also characterize the disease, and contribute to the gradual devastation and atrophy of AD brains (fig.2.1-b) (reviewed in 39).

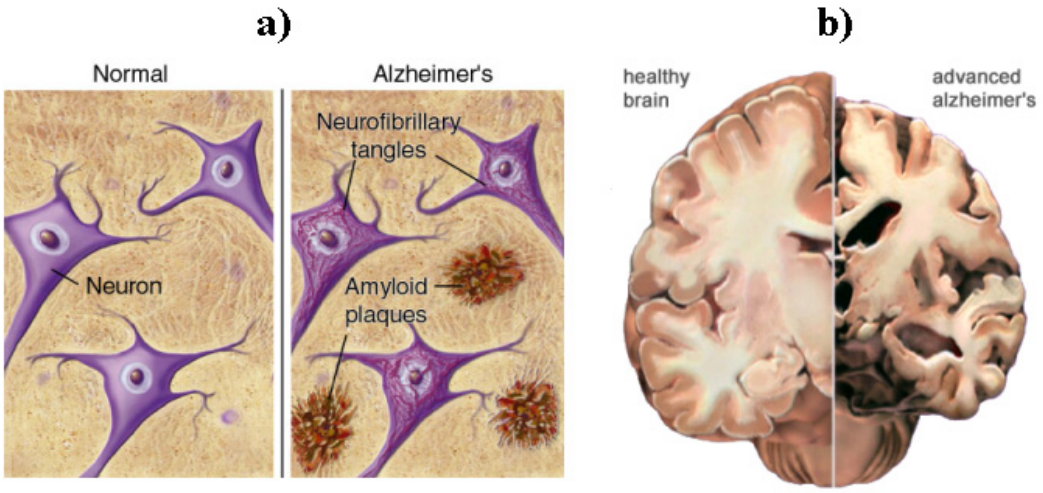

Figure 2.1: *a)* Illustration of two pathological features found in AD (right), SPs and NFTs. Picture drawn from (40). *b)* Cross-section of a healthy brain (left) versus an advanced AD brain (right) showing structural and brain volume changes. Used with permission from the Alzheimer's Association©2007 (41).

2.1.2.1 – Senile plaques (SPs) and beta-amyloid (Aβ) peptides

Although Aβ peptides and SPs can be found in healthy non-demented elderly subjects, a dysregulation of the amyloid metabolism appears central to the disease and is a prerequisite for AD diagnosis (2). An altered processing of the amyloid precursor protein (APP) results in an accumulation of the 39-43 amino acid forms of Aβ peptide [see section 2.1.3.1 for more details], which aggregate as soluble oligomers (2-10 units), enlarge and deposit as fibrils (100 units), and finally as SPs (Fig.2.2) (reviewed in 42, 43). These SPs are further classified as either immature/diffuse/amorphous plaques or mature/neuritic plaques. Diffuse plaques are the most prevalent type of SP found in AD (>80%, 44), and contain no fibrils or too few to be detected by Congo red. Neuritic plaques are further subdivided into cored and compact plaques; both contain fibrils, the former in

form of a central amyloid core, the latter without a core (Fig.2.3) (described in 45, 46).

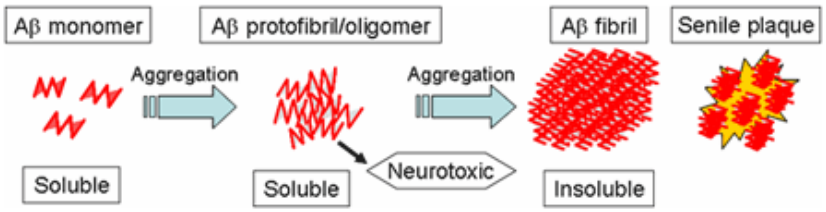

Figure 2.2: Model for the fibrillogenesis pathway of Aβ peptide. Illustration drawn from (47).

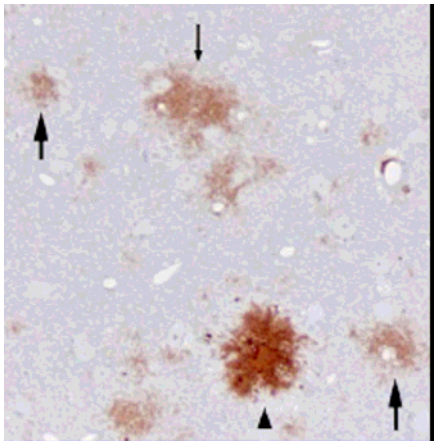

Figure 2.3: Diffuse plaques (large arrows), compact plaque (small arrow), and cored plaque (arrowhead) are shown from a semithin (0.5 um) resin section under a conventional bright field microscope. Photo drawn from (45).

The presence of SPs in the brain of all subjects suffering from AD led to the hypothesis that these extracellular deposits were responsible for the generation of NFTs and subsequent cell death through the induction of neurotoxicity (termed the *amyloid cascade hypothesis*, 48). However, a growing body of evidence now points to Aβ peptide oligomers as the primary toxic culprits in AD (reviewed in 42, 43). Of all types of Aβ peptide formed, it is the Aβ42 specie that is believed to be the most neurotoxic because it is far more prone to oligomerize and form fibrils than the more abundantly produced Aβ40 peptide (reviewed in 42, 43). While an increase in the levels of both Aβ40 and Aβ42 peptides are commonly found in AD, an increase in the ratio of Aβ42/40 is particularly noteworthy because it is associated with very early and aggressive forms of AD (49). Nevertheless, there still is a heated debate about whether the increase in Aβ levels

is a cause or a consequence of AD. Indeed, evidences indicate that the amyloid cascade hypothesis does not fully account for the pathology of AD. For example, a) some non-cognitively impaired individuals have both a high plaque burden and higher levels of Aβ peptide (50); b) increased levels of Aβ peptide have been observed following stresses such as traumatic head injury (51), suggesting that Aβ may be a consequence of cellular dysfunction rather than a causality; c) tangles and plaques can exist independently of each other (52); and d) the neurotoxicity of Aβ peptides, especially Aβ40, has not been convincingly proven because they were shown to be essential for neuronal viability, and proper synaptic function (53-56).

2.1.2.2 – Neurofibrillary tangles (NFTs)

NFTs are intraneuronal filamentous deposits that disrupt axonal transport and induce widespread metabolic decline, explaining around 85% of the neuronal loss observed in AD brains (57, 58) [see section 2.1.2.4 for additional details]. Because tangles and plaques can exist independently of each other (52), these two neuropathological features were believed to be largely independent from each other. However, recent evidences suggest that either APP or Aβ peptides can stimulate the formation of NFTs (59, 60), adding fuel to the fierce debate over Aβ as the primary cause of AD. Nevertheless, the hypothesis that the Aβ pathology is upstream and causes the formation of NFTs remains controversial given, among other things, the anatomical separation of SP and NFT deposition in AD brains (reviewed in 61). Indeed, the accumulation of NFT starts in the entorhinal cortex and hippocampus of the limbic system, it then spreads in the temporal lobe, then to the frontal cortex, and ultimately to primary sensory and visual areas (62-64). The precise and consistent development of NFTs allowed Braak and coworkers to established a six-stages classification (Braak I-VI) of neurofibrillary degeneration in AD (62, 63). This spatiotemporal mapping of NFTs in AD was later refined and expanded to ten stages (S1-S10) by Delacourte and colleagues (Fig.2.4) (64). For its part, even though the amyloid burden is much more diffuse, widespread, and heterogeneous in nature than the NFT distribution, five phases in SP

accumulation can be distinguished (65). SP deposition is initially restricted to the neocortex, it then spreads to the allocortical regions, advances to the diencephalic nuclei and striatum, then to specific brainstem nuclei, and finally to the cerebellum (Fig.2.5) (65). Despite their marked differences in spatiotemporal distribution, which obscure the proposed role of Aβ in NFT formation, SPs and NFTs might act synergistically, as NFTs were shown to require SPs to spread from stage S3 to S10 (Fig.2.4) (66).

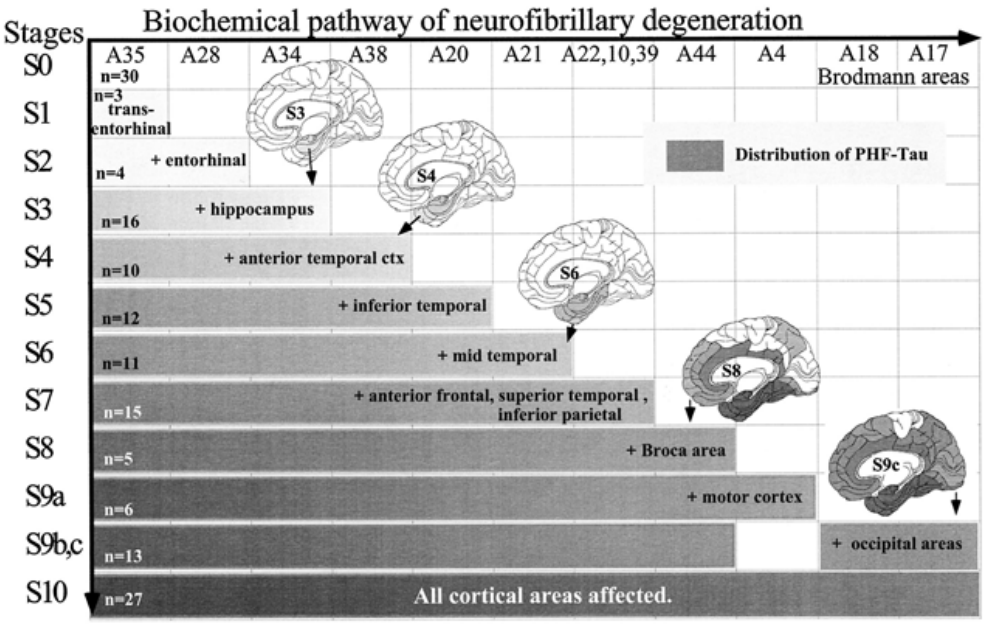

Figure 2.4: The biochemical pathway of neurofibrillary degeneration in AD. The brain areas affected at each stage of NFT deposition are depicted on the figure: *S0*, none; *S1*, transentorhinal cortex (Brodmann area [BA] 35); *S2*, entorhinal cortex (BA 28); *S3*, hippocampus; *S4*, anterior temporal cortex (BA 38); *S5*, inferior temporal cortex (BA 20); *S6*, mid temporal cortex (BA 21); *S7*, polymodal association areas, namely the anterior frontal cortex (BA 10), the superior temporal cortex (BA 22) and the inferior parietal cortex (BA 39); *S8*, unimodal association areas such as the Broca area (BA 40); *S9*, primary regions such as the occipital areas (BA 18 to 17), and the motor (BA 4) or sensory areas (BA 2, 3); and *S10*, all cortical and many subcortical areas. Figure drawn from (64).

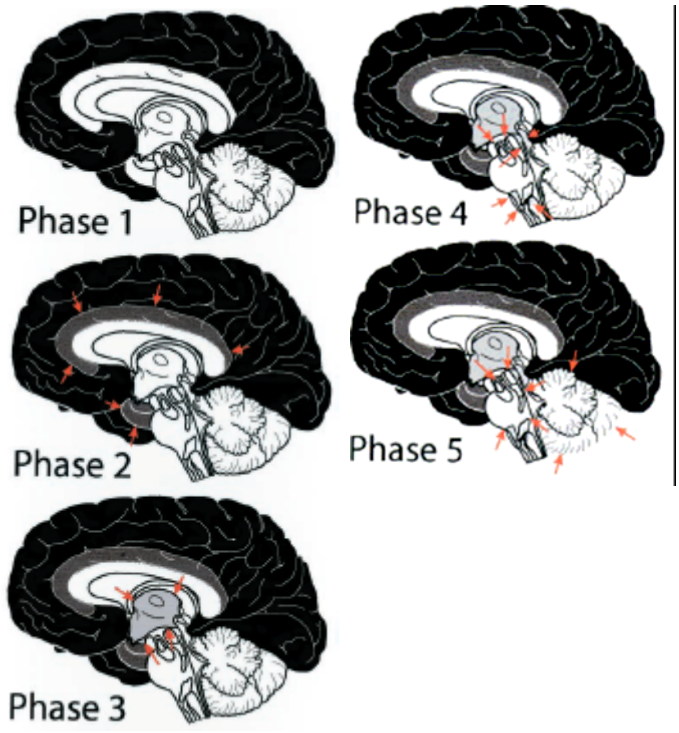

Figure 2.5: The 5 phases of Aβ deposition. The brain areas affected at each stage of Aβ deposition are depicted on the figure: *phase 1*, neocortex (black); *phase 2*, allocortical areas (red arrows) such as the entorhinal cortex, hippocampal Cornus Ammonis 1 (CA1) region, insular cortex, amygdala, cingulate gyrus and presubicular region; *phase 3*, striatum (not shown, caudate and putamen) and diencephalic nuclei (red arrows) such as the basal forebrain nuclei, thalamus and hypothalamus; *phase 4*, distinct brainstem nuclei such as the substancia nigra, red nucleus, central gray of the midbrain, CA4 and intermediate reticular zone; and *phase 5*, the cerebellum (red arrows) and additional brainstem nuclei (red arrows) such as the pontine nuclei, locus coeruleus, and oral and central raphe nuclei. Figure drawn from (65).

2.1.2.3 – Grey matter losses

Even though a gradual atrophy of the brain is observed, AD affects specific neuronal cell types and bypasses others (67-71). Of the two broad classes of cortical neurons, namely the local interneurons and the projection neurons, it is the latter that are exclusively lost in AD (68, 71, 72). Large projection neurons with short axons are spared late in AD, whereas those with long, thin and sparsely myelinated axons degenerate early in the course of the disease (reviewed in 68). There are several reasons why these particular neurons might be more vulnerable to AD, notably their high-energy requirement, their reliance on axonal transport for sustained function and trophic support, and their large and poorly protected

(sparsely myelinated) cell surface area that increases their exposure to toxic environmental conditions and oxidative stresses (67-69).

As often seen in neurodegenerative disorders, degeneration occurs preferentially in neuronal populations of particular neurotransmitter phenotype (69). In the case of AD, the neurons being killed in the greatest numbers are the neurons of the basal forebrain cholinergic system, especially those of the nucleus basalis of Meynert, which innervate the amygdala and neocortex (Fig.2.6a) (73, reviewed in 67, 74). Albeit to a lesser extent, other basal forebrain cholinergic neurons are lost in AD, including those of the medial septal nucleus, and horizontal and vertical diagonal bands of Broca, which predominantly innervate the hippocampus, olfactory bulb and anterior cingulate cortex, respectively (Fig.2.6-a) (73, reviewed in 67, 74).

The basal forebrain cholinergic neurons provide crucial inputs for all aspects of cortical function, especially in regard to attention, memory and emotions. Not surprisingly, the hippocampal network (Fig.2.6-b), which provides the key interconnection between the neocortex and hippocampus, a structure rich in cholinergic fibers and crucial for memory formation, is devastated in AD (reviewed in 67). Indeed, both the pyramidal neurons of the perforant path, which originate from the entorhinal cortex and terminate in the outer molecular layer of the dentate gyrus, and the output neurons of the hippocampal Cornus Ammonis 1 (CA1) region are extensively lost in the earlier stages of AD (Fig.2.6b) (reviewed in 67).

Numerous biochemical and *in situ* hybridization data also confirm the devastation of the cholinergic system in AD, notably the reported loss in both ChAT activity (from 30 to 95%) and mRNA levels (about 50%) in the temporal, frontal and parietal cortices of AD brains (75-77, reviewed in 74). Although ChAT is not rate limiting for ACh synthesis, its decreased activity is usually consistent with a reduction in the numbers of cholinergic neurons of the nucleus basalis of Meynert, and both features have been correlated with the degree of dementia (76, 78, reviewed in 74). Nevertheless, it remains to be proven whether

or not cholinergic neurodegeneration constitutes the pivotal functional determinant of the cognitive symptoms observed in AD (74).

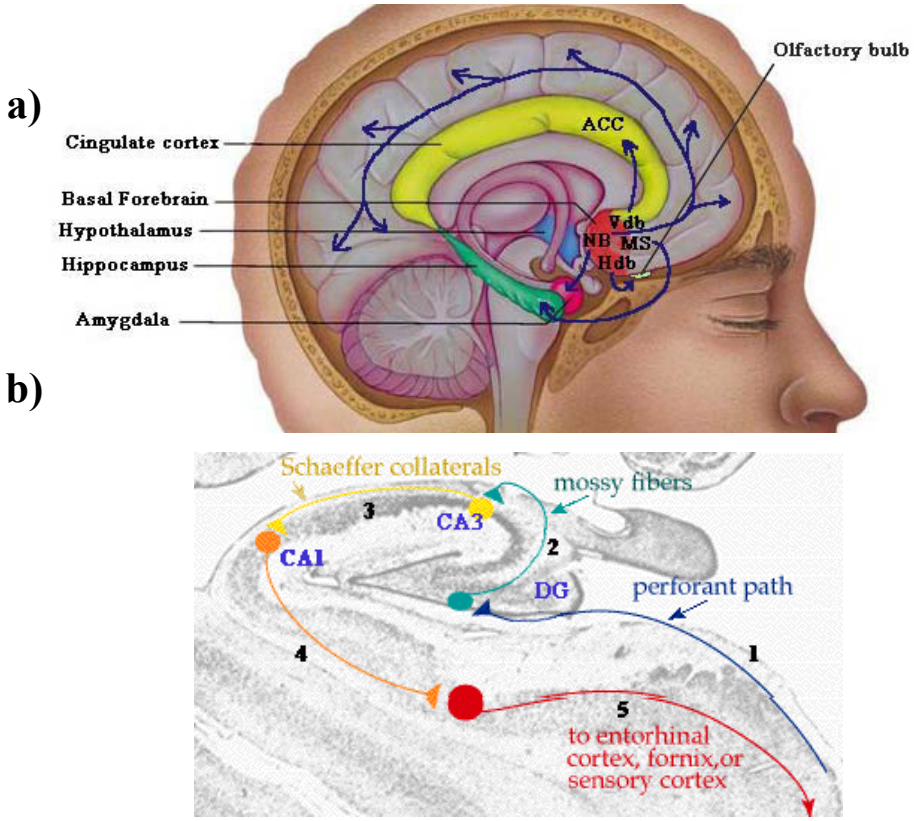

Figure 2.6: *a)* Illustration of the basal forebrain cholinergic system (red), which comprises the cholinergic neurons of the nucleus basalis of Meynert (NB), medial septal nucleus (MS), and horizontal (Hdb) and vertical diagonal bands of Broca (Vdb). The NB, MS, Vdb and Hdb cholinergic neurons innervate the neocortex and amygdala, hippocampus, anterior cingulate cortex (ACC), and olfactory bulb, respectively. *b)* Illustration of the hippocampal network: *1.* Via the perforant path, inputs from the entorhinal cortex terminate in the molecular layer of the dentate gyrus (DG). *2.* DG neurons then send axons to the neurons of the CA3 region via the mossy fiber pathway. *3.* These latter neurons then send axons to those of the CA1 region via the schaffer collateral pathway. *4.* These CA1 neurons then send the main outputs back to the neurons of the subiculum, *5.* which in turn forward the information to the entorhinal cortex or neocortex.

2.1.2.4 – Inflammation and oxidative stress

Few researchers will argue against the fact that enhanced oxidative stress and inflammation are primary events in the course of AD. Indeed, a plethora of oxidative reaction products has been found increased in the brains of AD and mild cognitive impairment subjects (79-83). This finding strongly suggests that

oxidative stress is involved in the earliest stages of AD, since mild cognitive impairment is a transition phase between normal aging and dementia (83).

As aforementioned, large neurons with long, thin and sparsely myelinated axons such as the cholinergic neurons of the nucleus basalis of Meynert are the most vulnerable cells to AD (68). These neurons also happen to contain the greatest numbers of lipofuscin granules (68) – organelles reminiscent of lipid peroxidation and composed of autofluorescent pigments and lipids (84) – indicating that they sustained extensive oxidative stress throughout their life. Interestingly, Braak and coworkers demonstrated that, even at old ages, neurons free of lipofuscin particles never develop AD-related pathology, regardless of whether their axon is protected by myelination (e.g. Betz cells, thick myelin sheet) or not (e.g. solitary cells of the Cajal, unmyelinated) (68, 85, 86). This finding suggests that oxidative stress may be one of the functional determinants of the development of AD-related pathology such as NFTs in neurons. Importantly, recent evidence indicates that oxidative stress, apotosis and excitotoxicity – the overstimulation of glutamate receptors leading to an excessive rise in intracellular calcium (Ca^{2+}) concentration and subsequent neuronal death – might also play a crucial role in inducing neuronal death without formation of NFTs (87). Oxidative stress thus appears as a key player in inducing neuronal death with or without (respectively 85 and 15% of the neuronal loss in AD) formation of NFTs in AD.

2.1.3 – Familial AD (FAD)

Although they all share the same neuropathological features, different etiologic forms of AD exist. The disorder can be subdivided into two broad categories, namely the familial (FAD) and sporadic (SAD) forms of the disease, which respectively account for about 2 and 98% of all cases worldwide (3). Because FAD and SAD usually strike someone in its fourth or fifth and seventh or eight decade of life, respectively, FAD is also named early-onset AD (EOAD), and SAD, late-onset AD (LOAD). When the first clinical symptoms of AD develop before age 65, subjects are classified as EOAD cases, will those who

have an age of onset of 65 years and older are considered as being of LOAD etiology.

FAD or EOAD cases can be elicited by inherited dominant mutations in the APP gene located on chromosome 21, presenilin (PS) 1 gene on chromosome 14 or PS2 gene on chromosome 1 (42). Interestingly, these three proteins are involved in the formation of Aβ peptides.

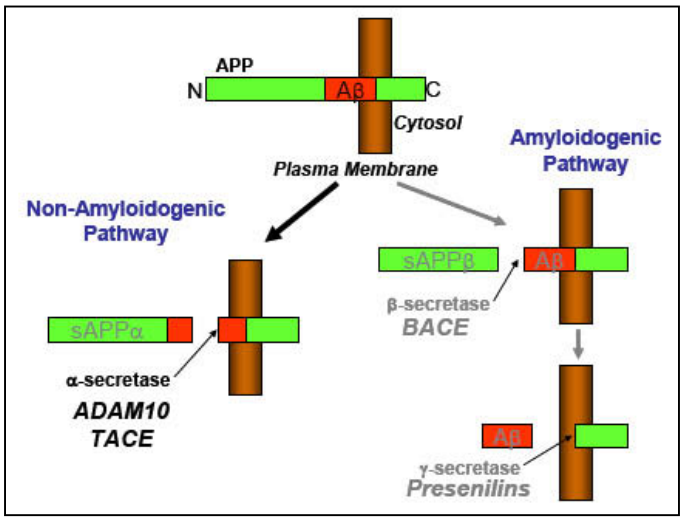

Figure 2.7: Processing of the APP via the amyloidogenic and non-amyloidogenic pathways. Non-amyloidogenic pathway (left): the APP is cleaved by the α-secretase, which precludes the formation of Aβ peptides. Amyloidogenic pathway (right): the APP is cleaved by the β- and γ- secretases to yield Aβ peptides (adapted form 88).

2.1.3.1 – Accent on the beta-amyloid (Aβ) metabolism

The processing of the APP can be performed via two pathways, namely the non-amyloidogenic and amyloidogenic pathways (Fig.2.7). The cleavage of the APP by α-secretase precludes the formation of Aβ peptides, whereas its consecutive cleavage by β-secretase and γ-secretase, which active site is primarily composed of PSs, generates two principal forms of Aβ peptide containing 40 or 42 amino acid residues depending on the exact point of cleavage by γ-secretase (Fig.2.7) (42, 88). A 50% increase in the production of Aβ peptides, especially of the Aβ42 form, is sufficient to elicit FAD (89, 90). The amyloid cascade hypothesis is thus derived from the work on FAD, and states all AD cases are caused by an increase in the levels of Aβ42, which is thought to trigger the

consecutive formation of SPs, NFTs, neuronal degeneration, and dementia (48). Numerous findings support the causative role of Aβ peptides in FAD (reviewed in 42). The strongest piece of evidence is that all Down's syndrome individuals who possess a third copy of chromosome 21, on which the APP gene is located, develop AD (91). However, even in FAD cases, clinical variations are common among affected individuals, providing evidence supporting a role for other factors in disease onset and progression.

2.1.4 – Sporadic AD (SAD)

In contrast, no inherited dominant mutations have been identified so far for SAD. Moreover, the observed greater levels of Aβ peptide in SAD do not seem to result, for the vast majority of cases, from an elevation in Aβ anabolism such as an increase in APP expression, or an overproduction of Aβ42 (90). An age-associated decrease in Aβ clearance, or in other terms, in Aβ catabolism, has been proposed as a better candidate mechanism to explain the observed increased levels of Aβ peptide and SP accumulation in SAD (92).

As demonstrated recently by Gatz and coworkers, the inheritance of the sporadic form of AD is considerably more complex than the familial subtype (93). Indeed, using more than 11000 pairs of twin, they showed that SAD results from interplay between genetic (75%) and environmental factors (25%) (93). There is thus an important genetic component to SAD. Accordingly, more than 350 candidate gene associations have been reported positive for SAD (94), but very few of them have been consistently confirmed in more than one population. The notable exception is the discovery of the association between apoE4 variant and SAD (4, 5). This genetic risk factor is now being recognized as the most important risk factor, second to aging, for both FAD and SAD.

2.1.4.1 – Aging, the most important risk factor for AD

About 5% of the population aged 65 to 74 suffers from SAD, whereas nearly half of those aged 85 and older are affected by it (2, 3). This brain disorder is therefore age-dependent, even in AD cases of the familial subtype.

How could aging predispose to AD? On the one hand, aging (see review by 95) is a biological process characterized by increased oxidative stress (96), accumulation of damaged proteins, lipids and nucleic acids (97, 98), increased levels of proinflammatory cytokines (99), decreased levels of neuroprotective factors (extensively reviewed in 95), and by a shift from adaptative, specific antibody-mediated immunity to innate, humoral and low-affinity antibody-mediated immunity (100). On the other hand, the CNS is characterized by a high oxygen consumption rate, high lipid content that is susceptible to lipid oxidation, and low levels of antioxidant enzymes compared with other organs (95, 101). As a consequence, the CNS is one of the most vulnerable organs to oxidative stress, and therefore to aging.

2.1.4.2 – ApoE4, the second most important risk factor for AD

Lipoproteins function to carry lipids across the body and are composed of both a hydrophobic core, which primarily contains cholesteryl esters and triglycerides, and hydrophilic layer, which contains numerous apos, enzymes, phospholipids and cholesterol molecules (Fig.2.8).

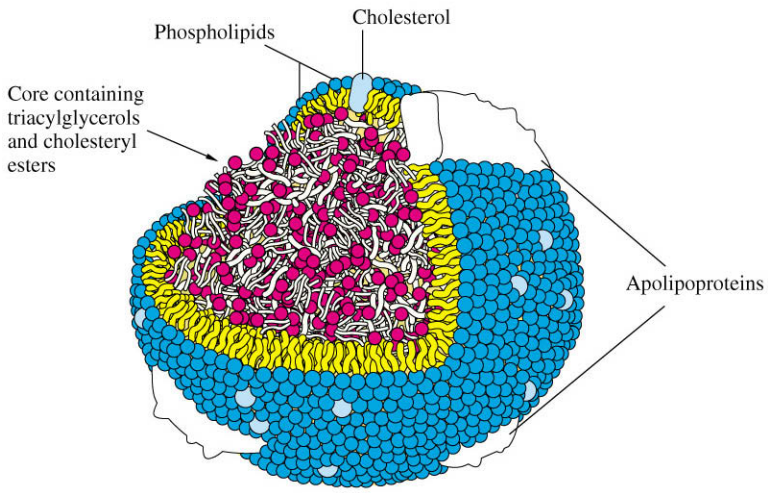

Figure 2.8: Illustration of a lipoprotein particle containing a hydrophobic core composed of triacylglycerols and cholesteryl esters, and a hydrophilic layer composed of apolipoproteins, phospholipids and free cholesterol. The presence of enzymes such as paraoxonase is not depicted on the figure.

Apos, such as apoAI, apoAII, apoB and apoE, are the major protein constituents of plasma lipoproteins, and serve as lipid solubilizers, receptor ligands and enzyme cofactors. Interestingly, apoE is the major apo in the CNS and plays a central role in lipid transport in the nervous system (102). The dependence of brain cells towards apoE as their most important lipid carrier and provider is emphasized by the complete absence of synthesis of other key plasma apos such as apoA1 and apoB in the CNS (103). ApoE gene is mapped on chomosome 19 and exists in humans as three possible isoforms that differ from each other by single amino acid substitutions at positions 112 and 158: E2 has two cysteines, E3 has both a cysteine (112) and an arginine (158), and E4 has two arginines. The apoE4 variant is the most prevalent genetic risk factor associated so far with the sporadic as well as the familial forms of AD (4, 5, 104-107), accounting for as much as 50% of the genetic variation in liability to develop the disease (108). Carriers of the apoE4 allele who develop AD, do so at an earlier age at onset, have higher levels of soluble Aβ peptide, increased SP and NFT accumulations, and more extensive cholinergic deficits (104, 106, 107). The effects of the apoE4 allele also appear additive such that heterozygotes and homozygotes, especially women, are three and eight times more likely to develop AD than non-apoE4 carriers. Interestingly, the apoE2 variant was shown to confer a marked risk reduction against SAD in multiple countries around the world (109). While the exact mechanism(s) responsible for the dual protective/risk effect of the apoE gene remain(s) elusive, the marked discrepancy that exists between the two isoforms could be due, at least in part, to their disparate levels in the brain and plasma, as apoE concentration was shown to follow a E2>E3>E4 gradient (110, 111). Hence, apoE2 is associated with the highest levels of apoE lipoprotein, whereas apoE4 is associated with the lowest.

2.1.4.3 – Accent on the brain lipid metabolism

How could lower levels of apoE lipoprotein particle enhance the risk of apoE4 carriers to develop AD? One must remember that lipids are vital constituents of cell membranes, and key components of the CNS. For instance,

cholesterol has been shown to be essential, among other processes, for axonal growth, and synaptic formation and remodeling (12, 13). These processes are crucial for learning, formation of memories and neuronal repair (12, 13), all of which are impaired in AD. Interestingly, two separate yet interrelated processes supply the brain cells in cholesterol: *de novo* synthesis, and internalization of lipoprotein particles. Because synapses and dendrites are located far away from the cell body where cholesterol synthesis takes place, the internalization of lipoproteins becomes imperative to meet the cholesterol needs required during dendrite sprouting, and synapse formation and remodeling (112). Lower levels of lipoproteins may therefore compromise the maintenance of synapse's integrity (112), explaining at least in part why the apoE4 variant confers a huge risk to develop AD.

2.2 – Brain lipid metabolism

The CNS contains a high concentration of lipids, second only to adipose tissue (113). Cells may contain up to 2000 different lipid species (113), two of which will be described in the following sections.

2.2.1 – Cholesterol, the CNS, and AD

The CNS is the cholesterol's richest organ in the body as it contains up to 25% of the total body's cholesterol, while representing only 2% of the total body mass (112). Several lines of evidence firmly indicate that cholesterol is of high relevance towards AD. For instance, the rate of synaptogenesis is decreased in AD, and studies point to cholesterol as the limiting factor of synaptogenesis *in vivo* (12, 13). Additionally, cholesterol may contribute to regulate inflammation, a primary event in the course of AD, as it serves as the precursor to all steroid hormones including corticoids, which regulate electrolytic balance, glucose metabolism and stress responses (14). Moreover, numerous studies suggest that cholesterol could regulate the formation of Aβ peptides and consequently, the formation of neurotoxic Aβ oligomers.

First, high levels of intracellular cholesterol result in an increase in Aβ generation and release (114, 115), whereas low levels of intracellular cholesterol (promoted by the use of statins which were shown to protect against AD incidence and/or progression (116, 117) favor APP processing via the non-amyloidogenic pathway (118, 119) (fig.2.7). Second, the generation of Aβ peptides is highly dependent on cholesterol because the cleavage of the APP by β-secretase is dependent on lipid rafts (88, 120), and the action of γ-secretase takes place within the cell membrane (121). Moreover, all the components of secretase enzymes are integral membrane proteins, and aberrant cholesterol transport has been shown to affect their subcellular distribution (122). Third, apoE was detected in SP and NFT lesions, suggesting that it might play a role in their formation. In that sense, apoE lipoprotein particles have been proposed to act as scavengers of soluble Aβ peptides (104, 106). Indeed, evidences suggest that apoE binds avidly to soluble non-aggregated Aβ fragments (123, 124). As it was demonstrated in rat primary neuronal cell cultures, the apoE lipoproteins containing Aβ may then be internalized via the apoE receptor internalization pathway (125, 126). Following internalization, these Aβ fragments could be released and degraded via the endosomal/lysosomal pathway (104, 106). The observation that Aβ reaches high intracellular concentration without affecting neuronal survival strengthens the proposed compartmentalization of internalized Aβ in endosomes/lysosomes (125, 126). Interestingly, apoE binding affinity for Aβ was shown to, again, follow a E2>E3>E4 gradient (127). This provides an additional mechanism explaining, at least in part, the marked discrepancy that exists between the E2/E4 variants and the risk to develop AD. Indeed, the protective apoE2 variant binds Aβ more avidly than the deleterious E4 variant, and might therefore be more efficient than its E4 counterpart at clearing Aβ fragments from the extracellular space (106).

2.2.2 – Phosphatidylcholine (PC), the cholinergic system, and AD

Cholinergic neurons have a unique need of PC that may contribute to their selective vulnerability in AD (128). These neurons use PC for two purposes: a) in

the composition of their cell membrane, given that PC is the predominant cell membrane phospholipid, and b) for the synthesis of ACh (129).

Briefly, ACh is synthesized from choline and acetyl-CoA by ChAT, and then packaged in pre-synaptic vesicles. Following stimulation, ACh is released into the synaptic cleft where it interacts with the nicotinic and muscarinic receptors of the post-synaptic neuron. ACh actions are then terminated by acetylcholinesterase (AChE), which hydrolyzes the remaining ACh to yield choline and acetate (130). In mammalian cholinergic neurons, choline, the rate-limiting molecule in ACh biosynthesis (131, 132), is supplied by three major sources. In order of importance, these three pathways are: 1) the uptake of free choline by low- and high-affinity transporters, 2) the cleavage of PC by, among others, phospholipase D, and 3) *de novo* synthesis (129-132).

Wurtman has previously proposed that AD might be associated with decreased uptake of choline in cholinergic neurons (128). In turn, these neurons might withdraw free choline from their membrane reservoir in PC (which accounts for 95% of the total choline pool), resulting in a decrease of membrane PC, cell shrinkage and ultimately, neuronal death (128). This autocannibalism might contribute to explain the selective vulnerability of cholinergic neurons in AD (128). Interestingly, the levels of glycerophosphocholine, a specific indicator of PC breakdown, have very recently been measured in the cerebrospinal fluid (CSF) of AD and non-demented age-matched control subjects (133). Glycerophosphocholine levels were strongly increased, by an average of 76%, in the CSF of AD subjects relative to non-demented controls, confirming that PC breakdown occurs in AD (133).

2.2.3 – Brain lipid homeostasis

Contrary to general belief, the brain is not a site of high lipid turnover. For example, the *overall* rate of cholesterol turnover in the CNS is only 1/20th the rate of turnover found in the whole body [0.03% vs 0.7%] (112). However, converging evidences suggest that lipids turnover rates vary considerably between different cell types and lipid pools within the CNS (reviewed in 112). According

to the rate of 24(S)-hydroxycholesterol excretion, the major pathway for removing cholesterol from the CNS, the rate of cholesterol turnover appears: a) exceedingly low in myelin, b) similar to other cells in the body in glial cells (0.08%), and exceedingly high (>20%) in particular types of large, metabolically active neurons such as the pyramidal cells of the cortex (112), which are lost in AD. This indicates that the maintenance of lipid homeostasis is of particular importance for the CNS, but most especially for neurons.

Lipid homeostasis is carefully maintained in the brain through a series of interdependent processes that include synthesis, transport, storage and degradation (134). Although cells composing the nervous tissue are capable of *de novo* synthesis of lipid molecules, they can also bind and take-up lipoproteins made available in the local environment for their lipid requirements (via receptor-mediated lipid internalization, also called the apoE/apoE receptor cascade) (134). Thus, lipid requirements of most brain cells are met by two separate yet interrelated processes: *de novo* synthesis, and internalization via the apoE/apoE receptor cascade (134).

2.2.3.1 – Cholesterol homeostasis in the CNS

The endogenous cholesterol synthesis pathway takes place in the cell bodies of brain cells, involves over 20 reactions, and is regulated primarily by the activity of the 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) enzyme, which catalyzes the formation of mevalonate, the key precursor molecule in the synthesis of cholesterol (Fig.2.11) (134). In the adult brain and under physiological conditions, brain cells, particularly astrocytes and neurons, synthesize cholesterol at a rate inversely proportional to the cholesterol content in the growth environment (134). As such, cholesterol biosynthesis, a high-energy cost reaction, is required only when lipoprotein internalization by the apoE/apoE receptor cascade is insufficient to meet the cholesterol requirement of the cell (135, 136). Indeed, in cells grown in excess of cholesterol-rich apoE lipoprotein particles, the HMGR activity was downregulated in favor of the lipoprotein internalization pathway (137, 138). A similar process has been reported in the

peripheral nervous system (PNS) and CNS during the acute phase of regeneration following experimental injury (139-141). As aforementioned, lipoprotein internalization by the apoE/apoE receptor pathway is mandatory to fulfill the cholesterol needs of neuronal structures located far away from the cell bodies [see section 2.1.4.3] (112). Synapses of distal axons such as those of the cholinergic neurons are thus highly dependent upon the internalization of lipoprotein particles to meet their cholesterol requirements.

2.2.3.2 – Choline and PC homeostasis in the CNS

In contrast, the two endogenous PC synthesis pathways, the choline and phosphatidylethanolamine *N*-methyltransferase [PEMT] pathways, take place both in the axons and cell bodies (142). Thus, under normal conditions, the internalization of lipoproteins is not necessary for distal axons to meet their PC requirements (143, 144). However, it is now becoming apparent that choline, the key precursor of PC, is an essential nutriment for both rodent and human life (142). To survive, animals must thus maintain a tight balance between gain and loss of choline (142). This is achieved through a series of three interrelated processes, namely a) choline acquisition by dietary intake and *de novo* synthesis, b) choline-recycling by PC/ACh breakdown and PC/choline redistribution, and c) choline depletion by choline oxidation, biliary PC excretion, and PC/ACh synthesis (142).

The impact of choline deprivation, which most likely occurs in SAD (128), was assessed using transgenic mice lacking the enzyme catalyzing the biosynthesis of choline and PC, PMET (142, 145). When fed on a choline-deficient diet, these mice are totally deprived of choline inputs and attempt to re-establish choline homeostasis in the liver and brain at the expenses of other tissues (142, 145). Of note, these mice boosted their choline-recycling pathways by increasing PC breakdown and initiating PC/choline redistribution, namely the transport of PC/choline from one tissue to another (142, 145). Although PC/choline redistribution was discovered in an extreme model, it most likely occurs during choline deprivation in wild-type animal and humans (142). In a

choline imbalance context such as SAD, the internalization of lipoprotein particles, the major carriers of cholesterol and PC in the body, might be necessary to meet the choline and PC requirements of brain cells, especially cholinergic neurons who have a unique need towards these molecules [see section 2.2.2].

2.2.3.3 – A model for brain lipid homeostasis

The integrity of large neurons with long axons such as the cholinergic neurons appears highly dependent upon the internalization of apoE lipoprotein particles, especially when the brain homeostasis is challenged such as during neurodegeneration, and synapse formation, plasticity and remodeling. To some extent, remodeling in the normal healthy brain may involve processes that parallel those involved in remodeling that occurs after injury (146). Synaptic remodeling following injury involves several key stages, and these are illustrated in figure 2.9.

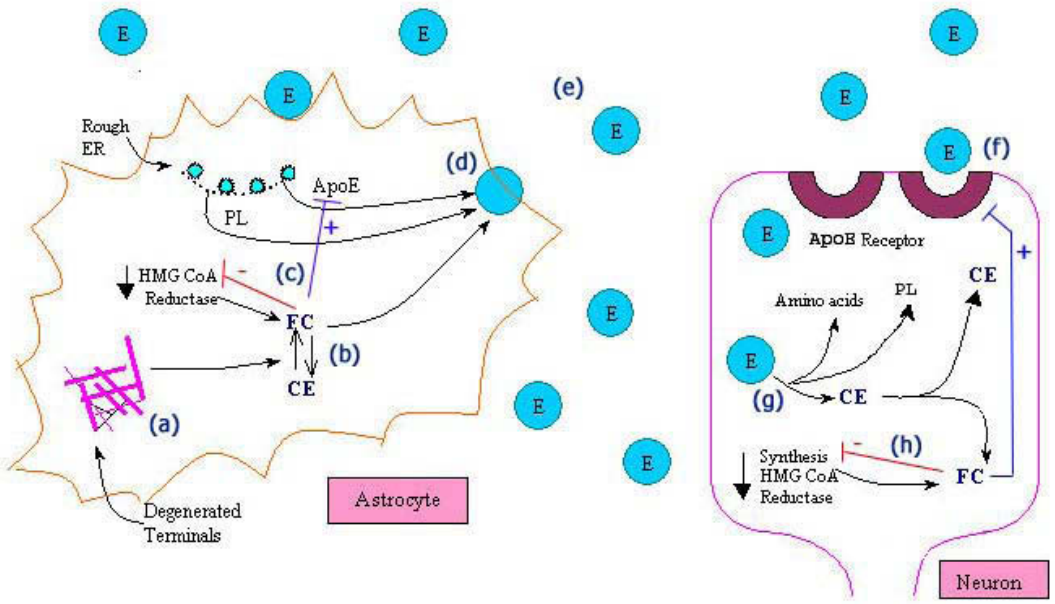

Figure 2.9: Stages of synaptic remodeling following injury. See text for details. FC, free cholesterol; CE, cholesteryl ester; PL, phospholipids; ER, endoplasmic reticulum; red line, downregulation; blue line, upregulation. Adapted from (147).

Ultrastructural studies using electron microscopy have shown that loss of neuronal inputs to the hippocampus of adult brains (a pathological feature of AD) prompts astrocytes, and to a lesser extent, microglia, to progressively engulf

degenerating axons (Fig.2.9-(a)) (147). Once metabolized, these terminals generate a large intracellular glial store of cholesterol readily available for the synthesis of membrane components necessary for the formation of new synapses and dendrites (Fig.2.9-(b)) (147). The intracellular accumulation of cholesterol in astrocytes inhibits *de novo* synthesis of cholesterol and triggers apoE synthesis (Fig.2.9-(c)), which, following complexion with cholesterol and phospholipids (Fig.2.9-(d)), will be secreted as a lipoprotein (HDL) in the extracellular space (Fig.2.9-(e)) (147). This lipoprotein will then be recognized by specific apoE receptors such as LDL receptor (LDLR) and apoE receptor 2 (apoER2) on the cell surface of neurons undergoing synaptic remodeling (Fig.2.9-(f)) (147). Following recognition and binding, the apoE/lipoprotein/apoE receptor complex is internalized and degraded, releasing cholesterol and phospholipids (Fig.2.9-(g)) (147). In response to the increased internalization of cholesterol via the apoE/apoE receptor pathway, neurons reduce their local HMGR activity by slowing down the energy-dependent *de novo* synthesis of cholesterol, and upregulate their expression of apoE receptors at the cell surface (Fig.2.9-(h)) (141, 147). This cholesterol-recycling model is consistent with the observed decline in *de novo* synthesis during active synaptogenesis (148).

In sum, internalization of lipoprotein particles via the apoE-apoE receptor pathway plays a pivotal role in regenerative and reinnervative processes in the CNS. Factors contributing to an optimal internalization of lipoprotein particles by cells may thus promote and preserve neuronal integrity, especially during aging, which is characterized by both a decline in biochemical processes including cholesterol biosynthesis and a concomitant increase in oxidative stress that promotes neuronal injuries. For instance,

- high concentration of circulating apoE lipoprotein particles,
- preservation of lipoprotein particles' integrity,
- and abundant presence of functional apoE receptors on the cell surface

could allow neurons, in their moments of greatest vulnerability, to efficiently meet their lipid requirements, especially cholesterol.

2.2.3.4 – AD, a disruption of brain lipid homeostasis

Not surprisingly, a compelling body of evidence strongly suggests a dysregulation of cholesterol metabolism in AD. In AD brains, numerous studies have reported decreased cholesterol levels in multiple brain areas (2, 149-151), increased demyelinating events resulting from lipidic alteration rather than myelin structure, notably in the frontal white matter (152), and decreased membrane width and cholesterol content in numerous brain regions (153). Importantly, numerous factors contributing to an efficient brain lipid homeostasis are perturbed in AD. Indeed, in AD brains (especially in SAD),

- the concentration of circulating apoE lipoprotein particles is reduced,
- the integrity of lipoprotein particles is compromised,
- and the abundance of apoE receptors on the neuronal cell surface is decreased.

First, the apoE4 variant, the most important genetic risk factor associated so far with AD, is associated with the lowest apoE levels in the CNS and plasma (110, 111). Second, the higher levels of oxidized proteins, advanced glycation end products, and 4-hydroxynonenal (HNE)-derived adducts that have been reported in AD brain tissue most certainly compromise the integrity of lipoprotein particles by saturating its antioxidant systems (79-83). Indeed, HDLs from the CSF of AD subjects are more easily oxidized *in vitro* than those extracted from the CSF of non-demented age-matched controls (154). Moreover, the oxidation of either HDL or LDL particles has been shown to induce degeneration of neuronal cells (155, 156), whereas the oxidation of HDL particles decreases the efficiency of its cholesterol transport by about 30% (157-159). Although the existence of oxidized-HDLs (oxHDLs) remains to be convincingly proven *in vivo*, converging evidences suggest that they are most likely present in tissues undergoing extensive inflammation and oxidation such as in AD brain tissues. Indeed, HDLs are easily oxidized *in vitro* , and undergo the same modifications as those observed in oxidized-LDLs (oxLDLs), whose existence *in vivo* has been proven (160). Moreover, receptors capable of binding oxHDLs are present in brain regions vulnerable to oxidative stress (161-164). Third, prevalent genetic variants in apoE

receptors such as LRP (9, 10) and SORL1 (11) have been reported to enhance the risk to develop SAD. The mechanisms mediating this risk enhancing effect remain elusive, since these receptors can interact with several proteins implicated in AD, including apoE, APP, and PSs. However, decreased levels of apoE receptors have been reported in AD brains relative to non-demented control brains (165), and this may compromise the efficient internalization of lipoprotein particles by neuronal cells.

Thus, the internalization of lipoprotein particles via the apoE-apoE receptor pathway appears compromised in AD, particularly in SAD. Given the importance of this pathway with respect to neuronal integrity and regenerative and reinnervative processes in the CNS, investigating the fate of genes involved in brain cholesterol transport and recycling has high relevance to AD. Among these genes, the human PON locus is of particular interest given its relevance towards not only the integrity of lipoprotein, but also towards the cholinergic system.

2.3 – Paraoxonase (PON)

2.3.1 – The PON family

The PON locus encodes three PON proteins, namely PON1, PON2 and PON3, and is located 5.5Mb downstream of the AChE gene and 150kb upstream of the pyruvate dehydrogenase kinase 4 (PDK4) on the long arm of chromosome 7 q21.3-22.1 in human (Fig.2.10) (166, 167). The three genes are well conserved among mammals and show high similarity in their structural characteristics; the three PONs share 65% identity at the amino acid level in a given specie, and 79-95% identity at the amino acid level and 81-95% identity at the nucleotide level between different species (168-170). Phylogenetic analysis revealed that the three PONs arise from gene duplication, PON2 being the oldest member, followed by PON3, and finally PON1 (170).

The name, paraoxonase, is derived from paraoxon, a toxic metabolite of the insecticide parathion, one of the first and most studied substrates of PONs. The name is now purely historical, as the PON family members have one of the broadest hydrolytic specificities known (171). It is becoming apparent that the

three PONs are lactonases with overlapping and distinct substrates specificities (172). PON1 also possesses modest arylesterase and low organophosphatase (OPase) activities, as it hydrolyzes a wide range of aromatic esters, and numerous insecticides and nerve agents (173, 174). For its part, PON3 possesses low arylesterase activity and almost no OPase activity, whereas PON2 possesses only lactonase activity (172, 175).

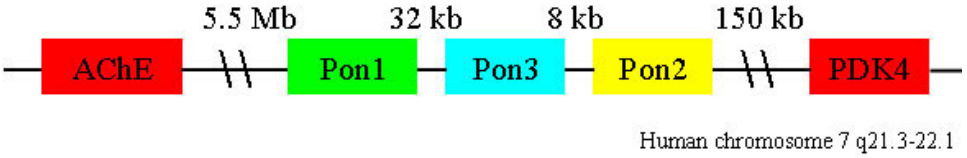

Figure 2.10: The three PONs are aligned next to each other on the long arm of chromosome 7 in human. AChE, acetylcholinesterase; PON, paraoxonase; PDK4, pyruvate dehydrogenase kinase 4; Mb, megabase; kb, kilobase. Adapted from (166).

An immunohistochemical analysis performed in 2008 in mice suggested an important protective and antioxidant role for the three PONs (176). Indeed, the three PON family members appear to play a central role in protection against environmental, prooxidative and toxic agents, free radical damages, and lipid peroxidation given their intense protein expression in, respectively, all epithelia (the point of entrance for pathogens and chemicals in the body), heart and skeletal muscles (tissues where the free radical production is intense as a consequence of high energy metabolism) and adipose tissues (the major storage site for lipids in the body), (176). However, the distribution of the three PONs was not identical, suggesting that they may not share exactly the same function (176). In contrast, the information about the tissue expression and distribution of the PON family members in humans is scarce, and only the Swedish Human Protein Atlas program has analyzed PON1 (but not PON2 or PON3) protein expression in different human tissues (data are freely available at http://proteinatlas.org/tissue_profile.php?antibody_id=1610). PON1 appears to be widely expressed in both mice and humans, and, within a given tissue, in equivalent cells (176), suggesting

that PON2 and PON3 protein expression in humans might share the same pattern as in mice.

PON1 and PON3 are associated mostly with HDL particles (177, 178). However, PON3 is about two orders of magnitude less abundant on HDL particles than PON1 (179). For its part, PON2 is membrane-bound and is not associated with lipoprotein particles (180). While PON1 and PON3 may act predominantly in the extracellular space, PON2 may exert its functions at the cellular level, joining the host of intracellular enzymes that protects the cells from oxidative stress (180). PON1 is by far the best-studied member of the family, and converging evidences suggest that it might play a role in AD.

2.3.2. PON1

The physiological roles of PON1 remain to be clearly established, but the enzyme appears to play a role both in the lipid metabolism and cholinergic system homeostasis.

2.3.2.1 – Role in the lipid metabolism

PON1 localization on HDL particles appears important of its activities *in vivo*, probably by stabilizing the enzyme and providing an optimal environment for the interaction with its physiological substrates (181, 182). Indeed, apoA1, the major apo of HDL particles in the periphery, stabilizes PON1 by over 300-fold and binds it with very high affinity (181, 182). Whereas PON1 arylesterase and OPase activities are only modestly affected (2-5-fold), the PON1 lactonase activity is stimulated by up to 20-fold by this association (181, 182). This suggests that the activities towards man-made chemicals, such as aryl esters and organophosphates (OPs), are promiscuous activities of PON1 rather than their primary functions (171). Despite its name, the primary function of PON1 is most likely to catalyze the hydrolysis of lactones, as well as lactone formation (172, 181-183). The fact that lactonase activity is the only activity common to all PON family members further emphasizes this hypothesis (181). Thus, lactones derived

from fatty acid oxidation products may comprise the native substrates of PON1 (172, 181).

There is ample evidence linking PON1 with the metabolism of lipids and oxidized lipids. PON1 was shown to protect against oxidative stress *in vivo* (19-24, 182), a phenomenon that can be attributed to its ability to metabolize oxidized lipids in both LDL and HDL particles (182, 184-186), in macrophages (21, 24, 182, 187, 188), as well as in atherosclerotic lesions (182, 189), and to its ability to protect lipids in LDL and HDL particles against oxidation (182, 190, 191). PON1 was also shown to inhibit the cholesterol accumulation in macrophages by several mechanisms, including the reduction of cellular intake of oxLDLs via the scavenger receptor CD36 (by hydrolyzing the lipid peroxides present on macrophages, PON1 prevents the upregulation of CD36 protein expression and consequently, the intake of oxLDLs via the CD36 pathway) (182, 187); the inhibition of cholesterol biosynthesis (182, 192); and the stimulation of cholesterol efflux (182, 193). Indeed, PON1 has been shown to inhibit, by about 40%, mouse macrophage cholesterol biosynthesis downstream of mevalonate at the lanosterol metabolic point (fig.2.11) (192). Moreover, studies using HDLs derived from wild-type, transgenic and PON1-knockout mice revealed that PON1 might act on macrophage phospholipids to form lysophosphatidylcholine, which in turn stimulates the HDL binding (by up to 60%) and HDL-mediated macrophage cholesterol efflux (by up to 40%) via the ABCA1 transporter (193).

Despite the ample *in vitro*, *in vivo* and *ex vivo* evidences for a role of PON1 in lipid metabolism, the biochemical activities mediating these functions remain elusive (182). Indeed, the observation of both PON1 phospholipase-A2 (PLA2) (194) and peroxireductase (195) activities was later ascribed to other serum enzymes contaminating the PON1 preparations (182, 196-198). On the other hand, highly purified PON1 preparations exhibited no antioxidant activity (198). However, a reconstituted *in vitro* system based entirely on purified apoA1 and lipid components, and recombinant PON1 was recently shown to inhibit LDL oxidation and to stimulate macrophage cholesterol efflux (182). Thus, highly purified PON1 preparations may remove PON1 from its natural environment and

impair its antioxidant functions, and the use of such preparations may be an inappropriate method with which to study the antioxidative functions of the enzyme (198).

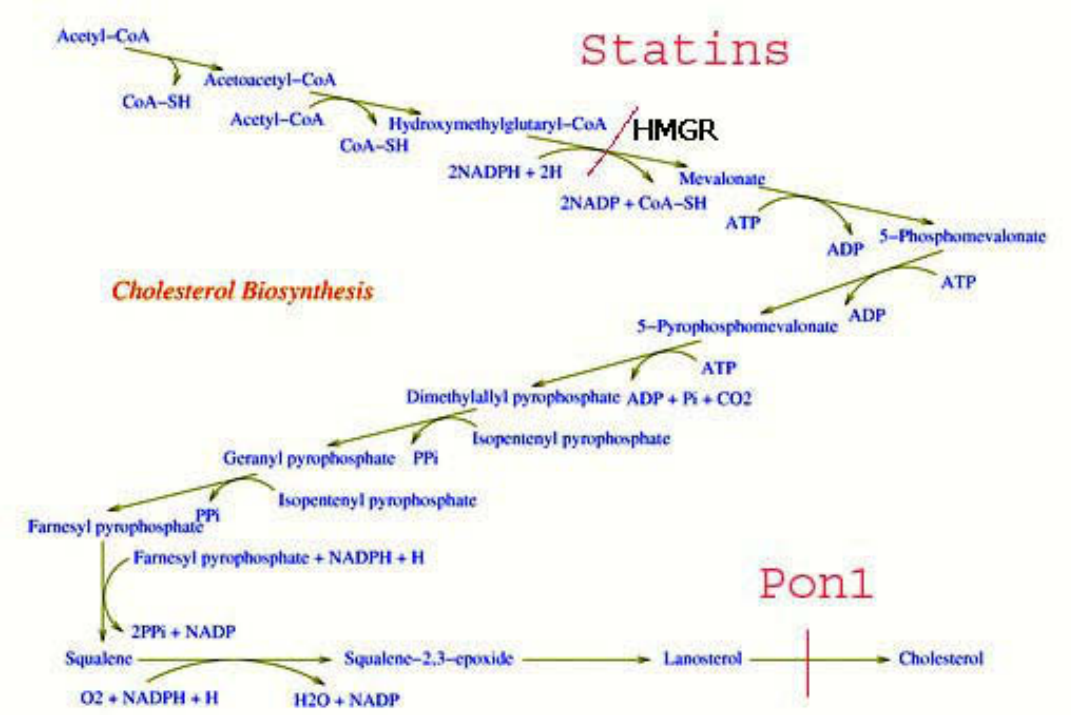

Figure 2.11: Cholesterol biosynthesis. The metabolic points where statins and PON1 inhibit biosynthesis are indicated in red (see text for details). Cholesterol biosynthesis is regulated primarily by HMGR, the enzyme that catalyzes the formation of mevalonate (see text for details). Adapted from www.cellml.org.

The recently solved crystal structure of PON1 led to a better understanding of the specific activity and catalytic mechanism probably mediating the lipid antioxidant properties of PON1 (171). Overall, PON1 is a six-bladed β-propeller enzyme, with each blade containing four strands (fig.2.12-a). In contrast to other β-propeller enzymes, PON1 has a closed active site defined by three α-helices, one of which contains the N-terminal signal peptide that is retained in the mature protein and allows the anchoring to HDL particles (HI on Fig.2.12-b,c) (171). The active site contains two Ca^{2+} atoms, one at the top (green) and one in the central section (red), which respectively serve catalytic and structural functions (Fig2.12-a,b,c) (171). The active site also contains many residues, two of which have been

extensively studied in the literature. The Q192R single nucleotide polymorphism (SNP) is located at the entrance of the active site, and the arginine (Arg, R) to glutamine (Gln, Q) substitution has been reported to affect OPase substrate specificity (10-fold decrease in paraoxon-hydrolyzing activity) (171). On the other hand, the leucine (Leu, L) to methionine (Met, M) substitution of the L55M SNP has been reported to affect the stability and enzymatic activity of PON1 (171). As highlighted by Harel and colleagues, this is due to the crucial role of Leu55 in packing the propeller's central tunnel and of its neighboring residues, which ligate both the catalytic and structural Ca^{2+} ions (171).

Site-directed mutagenesis revealed that both the catalytic Ca^{2+} ion and a histidine (His, H) dyad, composed of His115 and His134, mediate the lactonase and arylesterase activities, but not the OPase activity, of PON1 (171). Thus, these three activities share the same active site, but the residues involved in electron transfer during the enzymatic hydrolysis of OP differ from those mediating the arylesterase and lactonase activities (171). Interestingly, the PON1 H115Q and H134Q mutants exhibited diminished lactonase activity, reduced inhibition of LDL oxidation and decreased stimulation of cholesterol efflux (182). This suggests that the role of PON1 in lipid metabolism is mediated by its lactonase activity (182).

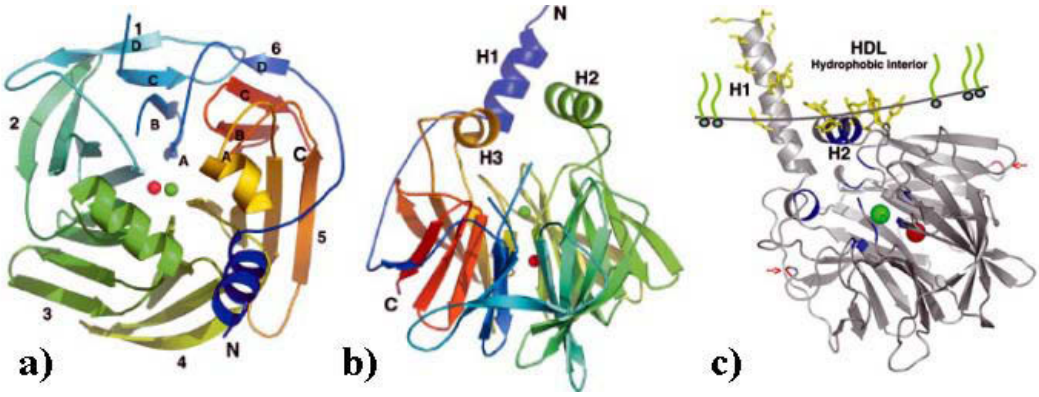

Figure 2.12: Overall 3D structure of PON1 protein. *a)* View of the six-bladed β-propeller from above. *b)* A side view of the β-propeller, including the three α-helices (H1-H3). *c)* Model of PON1-anchoring to a HDL. Shown are the two calcium atoms (green (top), catalytic; red (bottom), structural) and the N and C-termini. Figures drawn from (171).

2.3.2.2 – Role in the cholinergic system homeostasis

PON1 may also constitute a potent endogenous modulator of the cholinergic system. Indeed, OPs such as nerve agents, pesticides and paraoxon, which irreversibly and covalently bind to the active site of AChE and inactivate it, are bound reversibly and hydrolyzed by PON1 (166, 167). Interestingly, Bryk and colleagues demonstrated that healthy individuals had a parallel, age-dependent increase in AChE and PON1 arylesterase activities. Furthermore, carriers of the PON1 debilitating alleles (alleles associated with *decreased* PON1 activity) such as the L55M SNP displayed *increased* AChE activity (167). They proposed that the inverse relationship between PON1 and AChE activities might reflect several different levels of interaction (167).

At the genomic level, the age-dependent increase in AChE and PON1 arylesterase activities could be due to common regulatory elements that co-modulate the expression of these two genes, separated by only 5.5Mb (fig.2.10), in response to environmental stimuli (167) such as the increase in oxidative stress associated with aging. Indeed, the cholinergic system is known to be subject to complex expression regulation; for instance, ChAT and the vesicular ACh transporter were shown to share a common gene locus and regulatory elements for gene transcription (167, 199).

At the protein level, two different modes of interaction may be perceived (167). First, Byrk et al proposed that by reducing oxidative stress [see section 2.3.2.1], PON1 could protect AChE, a particularly sensitive enzyme inactivated under oxidative conditions (200), and prevent the overexpression of AChE-R, the AChE stress-induced splice variant (167). Reciprocally, low levels of PON1 protein could lead to an inactivation of AChE, which in turn elevates ACh levels and initiates AChE-R overproduction (167), explaining the increase in AChE activity observed in carriers of PON1 debilitating alleles. A second putative mode for AChE-PON1 interaction involves environmental exposure to low, residual doses of OPs, such as pesticides residues in fruits and vegetables, and domestic pesticides (167). Indeed, Byrk and coworkers proposed that residual doses of OPs in individuals with lower PON1 levels could upregulate AChE-R due to the

inhibition of circulating AChE (167). The importance of residual doses of OPs and PON1 as a protective enzyme against such agents was recently highlighted by a study performed on mothers with low PON1 activity and pesticide traces in their urine (167, 200). Indeed, these women gave birth to infant with smaller head circumference, predictive of subsequent cognitive defects (167, 200).

Individual carriers of the PON1 debilitating alleles might therefore have a more vulnerable cholinergic system, affecting individual susceptibility to experience-derived and environmental events such as the exposure to inhibitors of cholinesterase (ChEI) (167). Accordingly, Pola and coworkers demonstrated that subjects carrying an arginine allele at residue 192 of PON1 protein (Q192R SNP) are more likely to respond to ChEI therapy, the treatment of choice for mild-to-moderate AD, than their glutamine homozygous counterparts (201).

2.3.2.3 – PON1 as a genetic marker of AD?

As aforementioned, the internalization of lipoprotein particles via the apoE-apoE receptor pathway is vital for the neuronal integrity, and regenerative and reinnervative processes in the CNS. Converging evidences indicate that this pathway is highly compromised in SAD, and PON1 constitutes a potent candidate not only to protect its integrity but also to promote its induction. First, the antioxidative properties of PON1, which were clearly demonstrated in the periphery (19-24, 182, 184-191), could contribute to preserve the integrity of both the CNS lipoprotein particles and plasma membranes of neuronal and glial cells. Second, similarly to statins, PON1 could promote the internalization of HDL particles in neuronal cells undergoing remodeling. Indeed, statins represent a family of drugs that function to inhibit cholesterol biosynthesis through the inhibition of the HMGR enzyme (Fig.2.11), and they have been suggested to confer protection against the incidence and/or progression of AD (116, 117). It is hypothesized that low levels of statins penetrate the blood brain barrier and target the neuronal HMGR to reduce intracellular cholesterol biosynthesis, and upregulate the apoE receptor family members at the cell surface (134). This leads to a massive influx of cholesterol in neuronal cells through the induction of the

lipoprotein internalization pathway (134). Because PON1 has been shown to inhibit macrophage cholesterol biosynthesis downstream of mevalonate, at the lanosterol metabolic point (fig.2.11) (192), PON1, similarly to statins, might interfere with cholesterol biosynthesis in the CNS and promote the internalization of lipoprotein complexes via the apoE/apoE receptor pathway.

Moreover, the cholinergic system, which relies heavily on cholesterol and phospholipid homeostasis for proper function [see sections 2.2.2, 2.2.3.1, 2.2.3.2], is one of the earliest and most consistently affected neurotransmitter system in AD. The fact that PON1 might act as a crucial protective enzyme for the cholinergic system [see section 2.3.2.2, and (167, 201)] further emphasizes the relevance of investigating whether this enzyme is involved in AD etiopathology.

Accordingly, numerous PON1's polymorphisms have been identified, and their association with AD has been controversial as some studies demonstrate an association between some PON1's SNPs and increased risk to develop AD (25-33), whereas others failed to replicate these findings (34-38). These discrepancies may result from many factors, including the extensive linkage disequilibrium between SNPs within the PONs gene cluster, the observed ten-to-forty-fold variation in levels of PON1 protein and activity independent of genotype, the ethnic population and the size of the population used in theses studies (166). Furthermore, most of these studies were limited to the assessment of a genetic association between SNPs and AD, and no additional parameters such as the CNS Aβ levels, SP and NFT densities or ChAT activity were included. Thus, additional genetic association studies are needed to establish if PON1 is a genetic marker of AD. These studies should be perform at larger scale (use large sample size) and include core neuropathological markers of AD. Moreover, the observed ten-to-forty-fold variation that appears independent of genotype stresses the importance of determining PON1 activity and protein levels over straight genotyping.

The unexamined life is not worth living

Socrates

CHAPTER 3 – MAIN RESULTS

Chapter 3. Main results

Connecting text

Cholinergic neurons, which play a crucial role in learning and memory (202), may be one of the most active neuronal subtypes with respect to synaptic plasticity and remodeling (203). Given their long, thin and sparely myelinated axons, cholinergic neurons may indeed be more vulnerable to oxidative stresses and injuries, and consequently to undergo numerous synaptic remodeling episodes throughout their life. Cholesterol and phospholipid homeostasis, especially the internalization of lipoprotein particles via the apoE-apoE receptor pathway, appears vital for these regenerative and reinnervative processes in the CNS. As exhaustively described in chapter 2, converging evidence points to increased oxidative stress and disruption of lipid homeostasis as being crucial events implicated in AD etiopathology. It is thus not surprising that the cholinergic system is one of the earliest and most consistently affected neurotransmitter system in AD. Given its recognized protective role towards both the lipid and cholinergic metabolisms, PON1 might play an important role in AD etiopathology. We thus hypothesized that there would be positive associations between PON1 genetic variants and AD risk, onset, and progression. Therefore, we investigated the effect of two SNPs on AD risk and onset in a large homogeneous cohort of about 1000 subjects. We were also interested to see if these polymorphisms would ultimately impact on the neurological hallmarks of AD. Thus, we looked at the effect of PON1 genetic variants on Aβ levels, SP and NFT densities, as well as ChAT activity in multiple brains areas of AD and non-demented control subjects. The following manuscript was submitted to the *Human Molecular Genetics* journal.

Involvement of Paraoxonase 1 genetic variants in Alzheimer's disease etiopathology

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ABSTRACT

BACKGROUND: Evidences suggest that genes involved in brain cholesterol homeostasis are of particular relevance towards Alzheimer's disease (AD) etiology. Among these genes, paraoxonase 1 (PON1) has gained newfound interest from a public health perspective as recent studies suggest that PON1 L55M and Q192R genetic variants might affect individual susceptibility to environmental events, such as the exposure to cholinesterase inhibitors (ChEI). OBJECTIVE: ChEI therapy being the treatment of choice for mild-to-moderate AD patients, we sought to answer two main questions: a) are these genetic variants associated with increased AD risk, earlier age of onset/death, or shorter AD duration?; and b) are they affecting the neuropathological hallmarks of AD? METHODS: This genetic study used a large cohort of clinical and autopsy-confirmed AD cases and age-matched, cognitively intact control subjects from the Douglas Hospital Brain Bank, Quebec, Canada. RESULTS: Evidences presented here suggest multiple sex-specific effects of these polymorphisms on AD etiopathology. The L55M Met allele exerts an AD risk enhancing effect only in men ($p<0.01$), whereas both men and women carrying the Met55Met genotype exhibit an increased survival (3years, $p<0.05$) and a later age at onset of AD (2years, $p=0.074$). These genetic variants are also significantly associated, sometimes in opposite directions for both sexes, with beta-amyloid levels ($p<0.001$), senile plaque accumulation ($p<0.001$) and choline acetyltransferase activity ($p<0.05$) in respectively 2 out of 2, 5 out of 6, and 4 out of 6 brain areas. CONCLUSIONS: Our results suggest a possible involvement of PON1 in the etiopathology of sporadic AD.

INTRODUCTION

Alzheimer's disease (AD), the most common form of dementia in the elderly, is a progressive neurodegenerative disease that is etiologically heterogeneous. Core pathological correlates of the disease include neuronal and synaptic losses, as well as accumulation of senile plaques (SPs) and neurofibrillary tangles (NFTs). The inheritance of the common form of AD (also known as sporadic) appears considerably more complex than familial AD, and probably reflects the co-action or interaction of several genes with environmental factors (93). More than 350 candidate gene associations have been reported positive for sporadic AD (94), but very few of them have been consistently confirmed in more than one population. The notable exception was the discovery in 1993 of the association between apolipoprotein (apo) E4 allele and AD (4, 5) – a landmark association that has now been confirmed hundreds of times worldwide.

In addition to the E4 allele of apoE, the predominant lipid carrier protein in the central nervous system (CNS), a large cluster of genetic markers involved in cholesterol homeostasis has been associated with AD, including alpha$_2$-macroglobulin (6), lipoprotein lipase (7, 8), and apoE receptors such as LRP (9, 10) and SORL1 (11). Several other proteins known to be involved in cholesterol homeostasis in the cardiovascular system, and also present in the CNS, are now being investigated in the context of AD etiopathology. Among these genes, the human paraoxonase 1 (PON1) locus is of particular interest for two major reasons.

First, lipid oxidation, which has been shown to predispose to atherosclerosis (204, 205) and dementia (206), can modify the structure of proteins and severely impair their physiological functions. For example, the lipid peroxidation product 4-hydroxynonenal (HNE) was shown to impair glucose and glutamate transport (207, 208) as well as Na^+, K^+-ATPase activity (209, 210), and to mediate, at least in part, the neurotoxic effect of beta-amyloid (Aβ) peptide, the core component of SP seen in AD (209). These latter findings are of interest in relation to PON1, a high-density lipoprotein (HDL)-associated enzyme, because it contributes to prevent the oxidation and the lost of function of two major lipid

carriers in the body: the low-density lipoprotein (LDL) and HDL particles (184, 190, see review 211). Second, the cholinergic system, which relies heavily on cholesterol and phospholipids homeostasis for proper function (148), is the earliest and most consistently affected neurotransmitter system in AD. Interestingly, Byrk and coworkers have recently shown that healthy individuals carrying PON1 debilitating alleles (associated with decreased PON1 activity, such as the methionine allele at residue 55) might have a more vulnerable cholinergic system. Indeed, these individuals displayed an increase in acetylcholinesterase (AChE) activity resulting from the overexpression of their AChE *stress-induced* splice variant (167). This finding raises the possibility that PON1 genetic variants might affect individual susceptibility to experience-derived and environmental events, such as the exposure to cholinesterase inhibitors (ChEI) (167). Accordingly, Pola and colleagues demonstrated that subjects carrying an arginine allele at residue 192 of PON1 were more likely to respond to ChEI therapy, the treatment of choice for mild-to-moderate AD, than their glutamine homozygous counterparts (201). While little is known about PON1 functions in the CNS, these two sets of evidence, gathered in the cardiovascular system, suggest that PON1 may act as a protective enzyme for both the lipid and cholinergic metabolisms.

To address the issue of possible PON1 polymorphisms' contribution to AD etiopathology, we used the well-established association analysis approach (18). In this regard, a certain number of association studies, performed in majority in heterogeneous populations of the US and Europe, have investigated the possible implication of PON1 genetic variants in AD. However, the issue remains controversial as some studies demonstrate an association (25-33), whereas others failed to replicate this finding (34-38). Thus, for our study to constitute more than a mere replicate of known findings, we investigated a) the effect of PON1 genetic variants on core pathophysiological hallmarks of AD, and b) we took into consideration some well-known problems with association studies (18), and data analysis (212). Indeed, a number of factors bedevil genetic association studies and cause their poor or non-confirmation, such as genetic heterogeneity, statistical power, and population stratification (18). To circumvent some of these

confounding factors, we conducted our study in a large population of genetically homogeneous Quebec French Canadian (QFC) (213, 214). Affected individuals in this founder population, which are direct descendants of the first settlers who colonized Canada in the 17th century, have an increased chance to share the same susceptibility alleles inherited from a common ancestor (215). This approach allows to minimize background noise and to increase sensitivity. Additionally, as highlighted by Seda and colleagues, susceptibility to diseases may have both unisex and sex-specific genetic determinants (216), a situation which might be hided by adjustment for gender during the statistical analysis of data (212). Results presented here have therefore been thoroughly screened for gender-specific effect by including gender in statistical analysis as a cofactor rather than a covariate.

Evidences presented in this article suggest strong gender-specific associations between PON1 and AD etiopathology, as specific genetic variants were associated with a) increased liability to develop AD (men only), b) increased survival and age at which the first symptoms of AD are clinically observed (age at onset), c) increased ratio of Aβ42/40, d) increased (in men) and decreased (in women) SP accumulation in multiple brain areas, and e) decreased choline acetyltransferase (ChAT) activity in numerous brain areas.

RESULTS

Two common PON1 single nucleotide polymorphisms (SNPs) known to affect the coding portion of the gene were genotyped in a large homogeneous Eastern Canadian population (see demographic characteristics in Table 3.1). These two SNPs are non-synonymous (rs662, Q192R; rs854560, L55M) and were selected according to PON1 protein structure (171). The Q192R SNP is located at the entrance of the active site, and the arginine (Arg, R) to glutamine (Gln, Q) substitution has been reported to affect substrate specificity (10-fold decrease in paraoxon-hydrolyzing activity) (171). On the other hand, the leucine (Leu, L) to methionine (Met, M) substitution at the L55M locus has been reported to affect the stability and enzymatic activity (171). As highlighted by Harel et al, this is

due to the crucial role of Leu55 in packing the propeller's central tunnel and of its neighboring residues, which ligate both the catalytic and structural calcium ions (171).

AD risk

PON1 L55M and Q192R SNPs were genotyped in a total of respectively 1066 and 958 age-matched, autopsy-confirmed or clinical AD and control cases (Table 3.1). Similarly to the observed worldwide proportion of familial and sporadic AD cases (about 5% and 95%, respectively), 91% of the AD cases in our study population were of sporadic etiology (also refer to as late-onset AD (LOAD); criterion: age of onset ≥ 65years). Nevertheless, we performed two sets of analysis using DeFinetti program (see material and methods). The first set of analysis considered the entire study population (both early-onset (EOAD) and LOAD cases), and the second one focused only on LOAD cases. The observed genotype distribution of the Q192R SNP was consistent with Hardy-Weinberg equilibrium (HWE), whereas it was only consistent in the control group for the L55M SNP. Statistical tests selected to estimate the association between PON1 and AD risk were therefore not imposing the assumption of HWE (217).

While the Q192R SNP failed to be associated with AD (supplementary material, Table 3.1S), the Met allele of the L55M SNP was associated with an increased risk to develop AD (Table 3.2). When the entire study population is scrutinized, only the LM carriers were at higher risk to develop AD relative to LL carriers (odds ratio (OR) 1.319, $p<0.05$, Table 3.2). Interestingly, if only LOAD cases are considered, both the LM and LM+MM carrier groups were at higher risk to develop AD relative to LL homozygotes (OR 1.388 and 1.303, respectively, $p<0.05$, Table 3.2). However, in both populations, the association failed to reach significance in MM relative to LL carriers ($p>0.5$). Subsequent stratification by gender revealed that the association between the Met allele and an increased risk to develop AD was only significant for men (LM: OR 1.738, $p<0.01$; LM+MM: OR 1.614, $p<0.05$), especially in the LOAD population (LM: OR 2.003, $p<0.001$; LM+MM: OR 1.859, $p<0.01$; Armitage's trend test: OR 1.261, $p<0.05$, Table

3.2). Again, the association failed to reach significance in MM relative to LL carriers in both the EOAD/LOAD and LOAD male populations ($p>0.3$).

Age and duration

The effect of PON1 SNPs on age of death (N=517), age at onset (N=298) and duration of AD (N=298) was assessed in autopsy-confirmed and age-matched AD and control cases using Cox regression analysis. In the sample tested, we found no significant impact of the Q192R SNP on age of death, age at onset, or duration of AD ($p>0.05$, see supplementary material, Table 3.2S). However, Cox regression analysis revealed an impact of the L55M SNP on age of death (hazard = death) and age at onset (hazard = onset of AD symptoms), as male and female subjects carrying the MM genotype decreased their risk of death and of AD symptoms onset by respectively 31% (hazard ratio (Exp (B)) 0.689, $p=0.031$, Table 3.3) and 34% (Exp (B) 0.664, $p=0.074$, Table 3.3). These results suggest that MM carriers normally die older (+3.3 years according to Kaplan-Meier analysis, $p<0.05$) and, if affected by AD, they tend to exhibit a slightly later age at onset (+2 years according to Kaplan-Meier analysis, $p=0.074$). The plausibility of a dual effect of the MM genotype on delaying age of death as well as age at onset is consistent with the fact that this genotype had no impact on duration of AD (Exp (B) 0.931, $p>0.7$, Table 3.3).

Aβ levels

Impact of PON1 SNPs on Aβ levels was assessed in the frontal cortex (N=109) and hippocampus (N=40) of autopsy-confirmed and age-matched AD and control cases using multivariate analysis of variance (MANOVA). The Q192R genetic variants had a significant impact on Aβ levels in both brain areas studied (Fig.3.1), especially on the ratio of Aβ42/40. In the hippocampus, apoE4+ males who carry at least one Arg allele were shown to exhibit a decrease of the ratio of Aβ42/40 (8-fold decrease, $p<0.05$, Fig.3.1). In the frontal cortex, both males and females afflicted by AD and carrying at least one Arg allele were

shown to exhibit a decrease of the ratio of Aβ42/40 (3-fold decrease, $p<0.01$, Fig3.1).

The L55M genetic variants also had a significant impact on Aβ levels in both brain areas (Fig.3.2), especially on the ratio of Aβ42/40. Consistent with the effect seen on age of death and age at onset, both Met alleles at the L55M locus had to be present to significantly affect Aβ levels. In the hippocampus, while no significant impact of the L55M SNP on Aβ40 and Aβ42 levels was observed ($p>0.05$), male carriers of the MM genotype significantly exhibited a drastic increase of their Aβ42/40 ratio relative to male LL and LM carriers by respectively 14- and 7-fold ($p<0.001$, Fig.3.2). In the frontal cortex, regardless of gender, AD subjects carrying the MM genotype significantly exhibited: i) a 7-fold decrease of their Aβ40 levels relative to AD LL carriers ($p<0.05$), and ii) a 1,8-fold increase of their Aβ42 levels relative to AD LL and LM carriers ($p<0.05$, Fig3.2). However, in this same brain structure, only AD females carrying the MM genotype significantly displayed an increase of their Aβ42/40 ratio relative to AD females carrying the LL and LM genotype by 10- and 5-fold, respectively ($p<0.001$, Fig.3.2).

NFT and SP densities

Systematic analysis of the impact of PON1 polymorphisms on neuritic SP (diffuse plaques were purposefully excluded from our counts) and NFT densities, in modified Bielchowsky stained brain sections from 60 autopsy-confirmed AD cases in six different regions, was performed using MANOVA. Both Q192R and L55M polymorphisms appeared to have little or no significant impact on NFT accumulation (data not shown). In contrast, the L55M SNP definitively correlated with neuritic SP density in a gender-specific manner (Fig.3.3). The Met allele at the L55M locus was found to markedly affect, in opposite directions, neuritic SP accumulation in men and women (Fig.3.3). In men, carriers of the MM genotype had *more* neuritic SP than those carrying the LL genotype in two out of the six brain regions examined, respectively the fusiform gyrus (1.7-times more, $p<0.05$) and frontal cortex (2.4-times more, $p<0.05$, Fig.3.3). In addition, when the neuritic

SP counts from the six brain regions were pooled, mean and total neuritic SP densities were significantly increased in male carriers of the MM genotype relative to those carrying the LL genotype ($p<0.05$, Fig3.3). This suggests that in men, the impact of the L55M SNP on neuritic SP accumulation might be more widespread, extending beyond the fusiform gyrus and frontal cortex.

In women, the extent to which the Met allele at the L55M locus affects neuritic SP accumulation is striking. Indeed, women carrying at least one Met allele had 1.5- to 2.1-times *less* neuritic SPs than those carrying the LL genotype in five out of the six brain areas examined ($p<0.05$ to $p<0.001$, Fig.3.3). Moreover, mean and total neuritic SP densities were significantly decreased in female carrying at least one Met allele relative to those carrying the LL genotype ($p<0.001$, Fig.3.3).

ChAT activity

Finally, the impact of PON1 SNPs was assessed on ChAT activity in six brain regions of autopsy-confirmed, age-matched and post-mortem delay (PMD)-matched AD and control cases using MANOVA. While the Q192R SNP had no significant effect on ChAT activity (data not shown), a significant correlation was established between L55M genetic variants and ChAT activity in four out of the six brain regions examined, namely the temporal cortex ($p<0.05$), the hippocampus ($p<0.05$), the thalamus ($p<0.01$), and the caudate ($p<0.05$) (Fig.3.4). These results suggest a gene dose-dependent effect of the Met allele on ChAT activity, as male and female carrying the MM genotype had up to 4- and 3-times less ChAT activity than their LL and LM counterparts, respectively (see thalamus structure, $p<0.01$, Fig.3.4). While ChAT activity in the cerebellum, a region rarely affected by AD pathology, was not significantly associated with the L55M genetic variants, a clear trend towards a decrease of ChAT activity in Met relative to LL carriers was observed in the putamen, a region affected by AD pathology only in the later stages of the disease (Fig.3.4).

DISCUSSION

The main aim of this pathophysiological study was to investigate the role of PON1 genetic variants in sporadic AD. The first question we sought to answer was whether or not the two coding PON1 SNPs could constitute genetic risk factors for AD. Results indicate that LOAD men carrying one Met allele at the L55M locus double the risk of developing AD relative to Leu homozygotes ($p<0.001$, Table 3.2). In contrast, men carrying the Met/Met genotype are not at significantly higher risk to develop AD relative to those bearing the Leu/Leu genotype ($p>0.3$, Table 3.2).

One possibility to explain this finding stems from the fact that AD is an age-related illness. Mortality from vascular diseases is much higher among men than women around the age of 50 years, and decreased PON1 activity, to which the Met allele, and especially the Met/Met genotype, has been associated (167, 218-220), is a risk factor for these illnesses as well (221). As such, a significant portion of men carrying the Met/Met genotype may have died prematurely of vascular diseases or developed AD before 65years of age, explaining, at least in part, why a double dose of Met allele is less frequent than expected and deviates from HWE in the LOAD male population relative to the male non-demented control population. Young to middle-aged women, on the other hand, are traditionally much more resistant to cardiovascular diseases than their male counterparts (222).

However, whether the L55M locus is causally involved in the association between PON1 and AD risk remains to be established. Indeed, extensive linkage desequilibrium have been reported within as well as between the PON1 gene and two other PON genes located nearby, PON2 and PON3 (31, 223). To substantiate our findings of an association between the L55M locus and AD etiopathology, additional investigations were designed to include other markers in the region. Ten other SNPs located in the vicinity of the L55M locus were assessed in our cohort of subjects, two within the PON1 gene (-162A/G, Q192R), four in the PON2 gene (including A148G and C311S) and four in PON3 gene (including F21F and A99A). None were significantly associated with increased risk to

develop AD (see supplementary material, Table 3.3S). Many of these SNPs were, however, significantly associated with the neuropathological markers (data not shown, except for the Q192R SNP, see Fig.1), suggesting that defining haplotypes and using them instead of SNPs might reveal more significant and powerful associations between PON1 and AD.

As such, the present study cannot rule out the possibility of a more significant association between AD etiopathology and another nearby PON polymorphism not assessed in this study, such as the PON1 promoter –108C/T SNP (218, 220). However, the evidences gathered here suggest a functional involvement of the coding PON1 L55M SNP in the illness. Indeed, the association between the L55M locus and AD was not only highly significant between the Met allele and increased AD risk, but also between the Met allele and nearly all the neuropathological markers examined in our case/control cohort. Accordingly, the Leu to Met substitution at the L55M locus was previously shown to affect arylesterase activity and to be associated with decreased PON1 activity (167, 171, 218-221). The lower enzymatic activity driven by the Met55 allele is believed to be due, given the importance of Leu55 in stabilizing the protein 3D structure (171), to decreased levels of PON1 protein (167, 219). Nevertheless, in view of the strong impact of the Q192R SNP on Aβ levels (Fig.3.1), which remained significant after correcting for the L55M effect, we believe that, rather than alone, the Met allele at the L55M locus is acting in concert with other PONs' polymorphisms to influence AD etiopathology. This will be the subject of new biochemical studies in the near future.

That being said, could the Met allele be associated, as seen in the periphery (167, 171, 218-221), with decreased levels of PON1 protein and activity in the CNS of both control and AD subjects? If that proves to be true, our results would support a role for lipoprotein complexes as scavengers of normally secreted extracellular lipophilic/non-aggregated Aβ *in v ivo* (1, 106). Indeed, decreased PON1 activities could, as we proposed in previously published preliminary data (1), promote the generation of oxidized HDLs whose electrical charges will repulse lipophilic Aβ. This could result in diminished HDL scavenging function

leading to a local increase in Aβ levels (1, 106). In turn, this could lead to an increase in the formation of neurotoxic pre-fibrillar Aβ oligomers (1, 106), which are believed to be the primary toxic culprits in AD and composed almost exclusively of Aβ42 (42). In view of both the relative toxicity and propensity to aggregate of these molecules (42), this may result, respectively, in an increase in neuronal damage and SP accumulation in the brain of AD subjects.

This latter model is consistent with the data of male Met allele carriers, which showed: i) an enhanced risk to develop AD (Table 3.2); ii) increased levels of the more neurotoxic form of Aβ (increased frontal cortex Aβ42 levels and hippocampal Aβ42/40 ratio, $p<0.05$ and $p<0.001$, Fig.3.2); iii) an increase in neuritic SP accumulation (Fig.3.3); and finally iv), a decrease in ChAT activity (Fig.3.4), which is usually symptomatic of a compromised cholinergic system function. The absence of association between the Met allele and the intracellular-generated NFTs could, on the other hand, be explained by the fact that PON1 is HDL-bound and may act predominantly in the extracellular space.

The effects of the Met allele on AD etiopathology in women were similar to those observed for men, except for the data pertaining to AD risk (Table 3.2) and neuritic SP accumulation (Fig.3.3). Indeed, the association between the Met allele and enhanced risk to develop AD is not observed in women ($p>0.2$, Table 3.2). Similar gender-specific associations with AD have been reported elsewhere – such as the male-specific association between LDLR rs688 SNP and AD (224), and the observed lower AD risk odds ratio for apoE4 carrier males relative to apoE4 carrier females (225) – and our results add further support to the suggestion that AD genetic studies might benefit by analyzing males and females separately (212, 225).

The Met allele appears to be associated with a greater neurotoxic index in women, given the increased levels of the more neurotoxic form of Aβ (increased frontal cortex Aβ42 levels and Aβ42/40 ratio, $p<0.05$ and $p<0.001$, Fig.3.2), and the decreased ChAT activity in four out of the six brain areas examined (Fig.3.4). However, the Met allele was also associated with decreased, rather than increased, neuritic SP accumulation in women (Fig.3.3), a surprising finding given the

predominance of Aβ42 over Aβ40 species in the composition of SPs seen in AD (44, 226). Importantly, we must mention that the vast majority of SPs found in AD are of the diffuse type (around 85%, 44), and this type of SP was excluded (on purpose) from our counts. As such, our amyloid findings suggest that men and women might look alike when global measures are considered, such as the Aβ42 and Aβ40 levels (see frontal cortex, Fig.3.2), whereas they might differ when much more specific measures are scrutinized, such as the numbers of neuritic SP they possess (Fig.3.3). Interestingly, our neuritic SP counts significantly correlated with the Aβ42 levels in men, but with the Aβ40 levels in women, suggesting that the main component of neuritic SPs may differ between men and women. Accordingly, sex-related differences in Aβ levels and Aβ deposition have been reported in mouse models of AD such as in Tg2576 mice (APP-Swedish mutation, 227) and APP/PS1 double transgenic mice (APP-swe/ PS1 A246E mutation, 228). These mice showed an increase in the levels of Aβ40 and Aβ42 and in the numbers of SP they possess, with female mice bearing heavier amyloid burden and higher plaque numbers relative to their male counterparts (227, 228). Similar findings have also been recently reported in a large cohort of >5000 autopsy-confirmed AD cases (229). So far, gender-dependent differences in neuritic SP composition have never been properly documented, and our results further stress the importance of investigating whether profound differences exist between male and female with respect to Aβ pathogenesis.

In conclusion, our results support an important role for PON1 in the pathophysiology of sporadic AD, with a marked impact on the cholinergic system and amyloid metabolism. Evidences presented in this article suggest strong gender-specific associations between PON1 and AD etiopathology, and highlight the importance of further investigating both the role of PON1 and the impact of gender in AD genetic and functional studies.

MATERIAL AND METHODS

Study population

This genetic study used a cohort of both autopsy-confirmed and clinical AD cases and non-demented controls from the Douglas Hospital Brain Bank, Montreal, Canada. Disease state (control or AD) of all participants was determined by clinical neuropsychologists, and was based on the following criteria: AD cases had to fulfill the NINCDS-ADRDA criteria for probable or possible AD (230, 231) and to exclude other dementing disorders, whereas normal controls had to be free of neurological or psychiatric diseases. In addition, autopsy-confirmed AD cases had to meet the NINCDS-ADRDA neuropathologic criteria for definite AD (230, 231), whereas autopsy-confirmed control cases had to be free of brain structural lesions (tangle and plaque indices reading $<20/mm^3$ and $<10/mm^2$, respectively, in at least one hippocampal and isocortical section thoroughly screened for areas of maximal injuries). This study was approved by the Ethics Board of the Douglas Hospital Research Center and all participants or, where appropriate, his/her caregiver, signed an informed consent.

Initially, our study was based on subjects of confirmed QFC origin (N=860), but we extended our analyses to Quebec citizens of other origins (N=206) since both groups had similar PON1 genetic distribution. In men and women, there are no significance differences between AD cases and control subjects in regard to age of death or age at recruitment (for autopsy-confirmed and clinical subjects, respectively). Age at onset and duration of AD are similar for both male and female AD cases. ApoE genotypes distribution is similar to previously reported prevalence for Eastern Canadians, with a strong and significant enrichment of the apoE4 allele in AD cases for both male and female subjects ($p<0.05$, Table 3.1).

DNA extraction

For autopsy-confirmed cases, DNA was extracted from blood or brain tissues using respectively QIAamp DNA Blood Kit and DNeasy tissue kit from

QIAGEN. For clinical cases, DNA was extracted from isolated blood lymphocytes by automated DNA extraction (NA-1000, AutoGen).

ApoE and PON1 genotyping

Genotype assays were performed according to standard methods described elsewhere (5, 232). Primer pairs were synthesised by Sheldon Biotechnology Institute (McGill University), DMSO and dNTP mix were from GE healthcare, Taq DNA polymerase, $MgCl_2$ and 10X PCR buffer were from QIAGEN (kit cat#201207), and restriction enzymes were from New England Biolabs (AflIII, HaeII, AlwI) and Promega (Hsp92II). Amplifications were carried out in a Perkin-Elmer PCR thermocycler, and digestion patterns were analyzed by viewing ethidium bromide/UV trans-illumination fluorescence on a Kodak DS 440 Imager.

Briefly, ApoE genotypes were determined using the following allele specific primers: forward 5'-CACGGCTGTCCAAGGAGCTGC-3' and reverse 5'-GCCCCGGC-CTGGTACACTGCCA-3'. Reaction was carried out in a volume of 20µl containing 500ng of DNA, 0.5 µM of forward and reverse primers, 0.2µl of 20mM dNTP mix, 2µl of 10X PCR buffer, 2µl of DMSO, and 0.4µl of Taq DNA polymerase. PCR cycling conditions began with a 5 min hot start at 94°C, followed by 35 cycles of amplification (94°C for 30s / 60°C for 30s / 72°C for 30s), and ended with a 5min extension cycle at 72°C (total of 37 cycles). PCR products were digested with both AflIII and HaeII and analyzed on 8% acrylamide gels.

PON1 L55M (rs854560) amplification was performed with the following primer pair: forward 5'-TTTCCATATAATCGCATTCATCA-3' and reverse 5'-GGGCATGGG-TATACAGAAAGC-3'. Reaction was carried out in a volume of 50µl containing 750ng of DNA, 10 pmol of forward and reverse primers, 1µl of 20mM dNTP mix, 5µl of 10X PCR buffer, 2µl of 25mM $MgCl_2$ (for a total $MgCl_2$ concentration of 4mM), and 0.5µl of Taq DNA polymerase. PCR cycling conditions began with a 10 min hot start at 95°C, followed by 35 cycles of amplification (95°C for 1min30 / 51°C for 2min30 / 72°C for 3min), and ended

with a 10min extension cycle at 72°C (total of 37 cycles). PCR products were digested with Hsp92II and analyzed on 2,4% agarose gels.

PON1 Q192R (rs662) amplification was performed with the following primer pair: forward 5'-TGCAGTTTGAATGATATTGTTGC-3' and reverse 5'-CTCCTGAGA-ATCTGAGTAAATCCA-3'. PCR conditions were identical to those used for L55M, except that the final $MgCl_2$ concentration in the reaction mixture was 2mM, and the digestions were performed with AlwI.

Homogenization and protein content determination

Brain regions of interest were dissected on dry ice and kept at -80°C until use. Frozen brain tissues were homogenized by sonicating samples for 2–3 bursts of 20s, on ice, using a minimal volume of phosphate saline buffer (10mM KPO_4 / 10mM KCl). Brain homogenate protein content was quantified by using the BCA protein assay (Pierce) using bovine serum albumin (BSA) as standard (Sigma).

Aβ peptide quantification

Aβ peptide levels in the hippocampus (comprising areas CA1 to CA4 and dentate gyrus) and frontal cortex (Brodmann area 9) were determined as previously described (233). Briefly, brain homogenates were incubated with gentle agitation in 5M guanidine hydrochloride solution. Samples were then diluted 1:10 in a protease inhibitor cocktail before centrifugation at 16 000*g* for 20min at 4°C. Aβ standards were prepared such that the final concentrations included 0.5 M guanidine and 0.1% BSA. Both Aβ40 and Aβ42 were quantified using sandwich ELISAs with a biotinylated monoclonal detection antibody (6E10, Senetek). Assay-specific, capture antibodies R165 and R163 (both generous gift from Dr P. Metha, NY, USA), which respectively recognized Aβ42 and Aβ40, were used to coat 96-well microtiter plates. Fluorescence was determined using a FL600 Microplate fluorescence reader (Bio-Tek Instruments). The sensitivity of both assays was approximately 100pg/ml.

ChAT activity

ChAT activity in the temporal cortex, hippocampus (comprising areas CA1 to CA4 and dentate gyrus), caudate, putamen, thalamus (comprising all thalamic nuclei) and cerebellum regions were determined as previously described (234). Briefly, brain homogenates were incubated for 15 min in buffer containing [^{14}C] acetyl-CoA, and radioactivity was quantified using a Beckman model LS7000 scintillation counter.

NFT and SP densities in AD

A group of 60 autopsy-confirmed AD cases underwent fine neuropathological mapping of the NFT and SP densities in six distinct brain areas: CA1 region of the hippocampus, subiculum, parasubiculum, fusiform gyrus, frontal cortex and parietal cortex. The neuropathological staining protocol was performed as previously described (75) and was consistent with the criteria used in the classification of Khachaturian (230). Briefly, paraffin-embedded sections were stained with haematoxylin and eosin, and modified Bielchowsky stain, and alkaline Congo red to visualize NFTs and SPs. Using a micrometric scale for calibration, readings were performed with a 10X objective for SPs and a 25X objective for NFTs. Diffuse plaques were ***excluded*** from all measurements. Screening of alkaline Congo red stains under polarized light was used to control for the reliability of tangle staining and, to a lesser extent, the SPs' affinity for the modified Bielchowsky preparation.

Statistical analyses

Associations between disease status (control vs AD) and PON1 SNPs were assessed using computer program DeFinetti (http://ihg2.helmholtzmuenchen.de/cgi-bin/hw/hwa1.pl), which computes various statistical tests for deviation from HWE and for phenotype-gene association. Controlling for ethnic origin, gender, apoE4 presence (E4 vs non-E4 carriers) and disease status (control, EOAD, LOAD), the effect of PON1 SNPs on age at onset, age of death, and duration of AD was evaluated by means of Cox regression analysis (by SPSS).

All the neuropathological sets of data presented here were analyzed using SPSS and EXCEL programs. The effect of SNPs on Aβ levels, SP and NFT densities as well as ChAT activities was assessed using MANOVA. In these analyses, sex, disease status and ApoE4 presence were included as cofactors, whereas ethnic origin was included as a covariate. Significant interactions between cofactors were analyzed using simple main effect tests, and main effects were analyzed using Tukey's Post Hoc-pairwise comparison tests.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST STATEMENT

None

LEGENDS TO FIGURES

Figure 3.1: Effect of PON1 Q192R variants on Aβ levels in the hippocampus (N=40) and frontal cortex (N=109) of autopsy-confirmed AD cases and age-matched controls. Data contrast carriers of at least one Arg allele (1) from those bearing the QQ genotype (0), and are expressed as pg/mg protein ± SEM. In the hippocampus, data are split according to gender (male/female: black/grey bar) and apoE4 allele presence (apoE4=0 vs at apoE4=1or2). In the frontal cortex, data are split according to disease status (control vs AD). Significantly different * $p<0.05$, **$p<0.01$ (MANOVA, Tukey's Post-Hoc).

Figure 3.2: Effect of PON1 L55M variants on Aβ levels in the hippocampus (N=40) and frontal cortex (N=109) of autopsy-confirmed AD cases and age-matched controls. Data contrast carriers of 0, 1 or 2 Met alleles, and are expressed as pg/mg protein ± SEM. In the hippocampus, data are split according to gender (male/female: black/grey bar). In the frontal cortex, data are split according to disease status (control vs AD) and, for the Aβ42/40 ratio, by gender as well (male/female: black/grey bar). Significantly different * $p<0.05$, **, $p<0.01$, ***$p<0.001$ (MANOVA, Tukey's Post-Hoc).

Figure 3.3: Effect of PON1 L55M variants on neuritic SP accumulation in the hippocampal CA1 region (CA1, N=33male/26female), subiculum (Sub, N=31/24), parasubiculum (pSub, N=34/26), fusiform gyrus (FG, N=33/26), frontal cortex (FCx, N=32/26) and parietal cortex (PCx, N=34/26) of autopsy-confirmed AD cases. Data are expressed as SP count/mm^2 ± SEM. For male, data contrast carriers of 0, 1 or 2 Met alleles. For female, data contrast carriers of at least one Met allele (1) from those bearing the LL genotype (0). Diffuse SPs were purposefully ***excluded*** from the counts. Significantly different * $p<0.05$, ** $p<0.01$, ***$p<0.001$ (MANOVA, Tukey's Post-Hoc).

Figure 3.4: Effect of PON1 L55M variants on ChAT activity in the hippocampus (Hippo, N=54), temporal cortex (TCx, N=36), caudate (Cau, N=20), putamen (Put, N=22), thalamus (Thal, N=13) and cerebellum (Ce, N=20) of autopsy-confirmed AD cases and age-matched controls. Data are expressed as nmol ACh/mg protein/h ± SEM, and contrast carriers of 0, 1 or2 Met alleles. Mean PMD varies between 20 and 30hrs and has no significant impact on ChAT activity. Significantly different * $p<0.05$, ** $p<0.01$ (MANOVA, Tukey's Post-Hoc).

TABLES

Table 3.1: Population demographics

	Study population		Men		Women	
	Control	AD	Control	AD	Control	AD
	(434)	(632)	(211)	(264)	(223)	(368)
QFC, # (%)	393 (91)	467 (74)	182 (86)	176 (67)	211 (95)	291 (79)
Age D/R, mean ± SD	74.5 ± 11.5	77.5 ± 9.2	73.6 ± 11.4	76.2 ± 9.0	76.0 ± 11.5	78.5 ± 9.0
Age at onset, mean ± SD	-	72.5 ± 8.5	-	71.9 ± 8.0	-	72.9 ± 8.8
AD duration, mean ± SD	-	7.1 ± 4.7	-	6.4 ± 4.3	-	7.9 ± 4.9
ApoE4 carrier, %	24	61	27	61	21	61

Abbreviations: QFC, Quebec French Canadian ethnicity; Age D/R, age of death or at recruitment; SD, standard deviation; AD, Alzheimer's disease; ApoE4, apolipoprotein E4

Table 3.2: Tests for association between PON1 L55M genetic variants and AD risk (in autopsy-confirmed and clinical cases)

		Overall Effect			Men			Women		
AD	Tests for association	N	p	OR	N	p	OR	N	p	OR
EOAD/LOAD	LL vs LM	1066	**0.039**	**1.319***	475	**0.006**	**1.738****	591	0.762	1.057
	LL vs MM		0.506	0.864		0.615	1.168		0.231	0.683
	LL vs LM/MM		0.105	1.234		**0.013**	**1.614***		0.982	0.996
	AT		0.602	1.003		0.125	1.170		0.517	0.882
LOAD	LL vs LM	973	**0.019**	**1.388***	430	**0.001**	**2.003*****	543	0.770	1.056
	LL vs MM		0.770	0.935		0.363	1.343		0.330	0.729
	LL vs LM/MM		**0.049**	**1.303***		**0.002**	**1.859****		0.986	1.003
	AT		0.369	1.043		**0.045**	**1.261***		0.607	0.901

Abbreviations: EOAD, early-onset AD (<65years); LOAD, late-onset AD (≥65years); AT, Armitage's trend test; OR, odds ratio; p, level of significance; L, leucine; M, methionine

Table 3.3: Cox regression analysis of the effect of PON1 L55M genetic variants on age at death/onset, and AD duration (in autopsy-confirmed cases)

Variable (hazard)	N	# Met	Exp (B)	p	Conclusion
Age of death	517	0	-	0.085	Met/Met carriers
(death)		1	0.873	0.162	significantly die
		2	**0.689**	**0.038***	older
Age at AD onset	298	0	-	0.202	Met/Met carriers
(onset of symptoms)		1	0.937	0.605	develop AD at
		2	**0.664**	**0.074**	older age [trend]
Duration of AD	298	0	-	0.414	Met/Met genotype
(death)		1	0.844	0.187	has no impact on
		2	0.931	0.758	duration of AD

Abbreviations: N, number of cases; Exp (B), predicted change in the hazard rate; p, level of significance

Disease, apoE4, gender and ethnicity status were included in the analysis

FIGURES

Figure 3.1

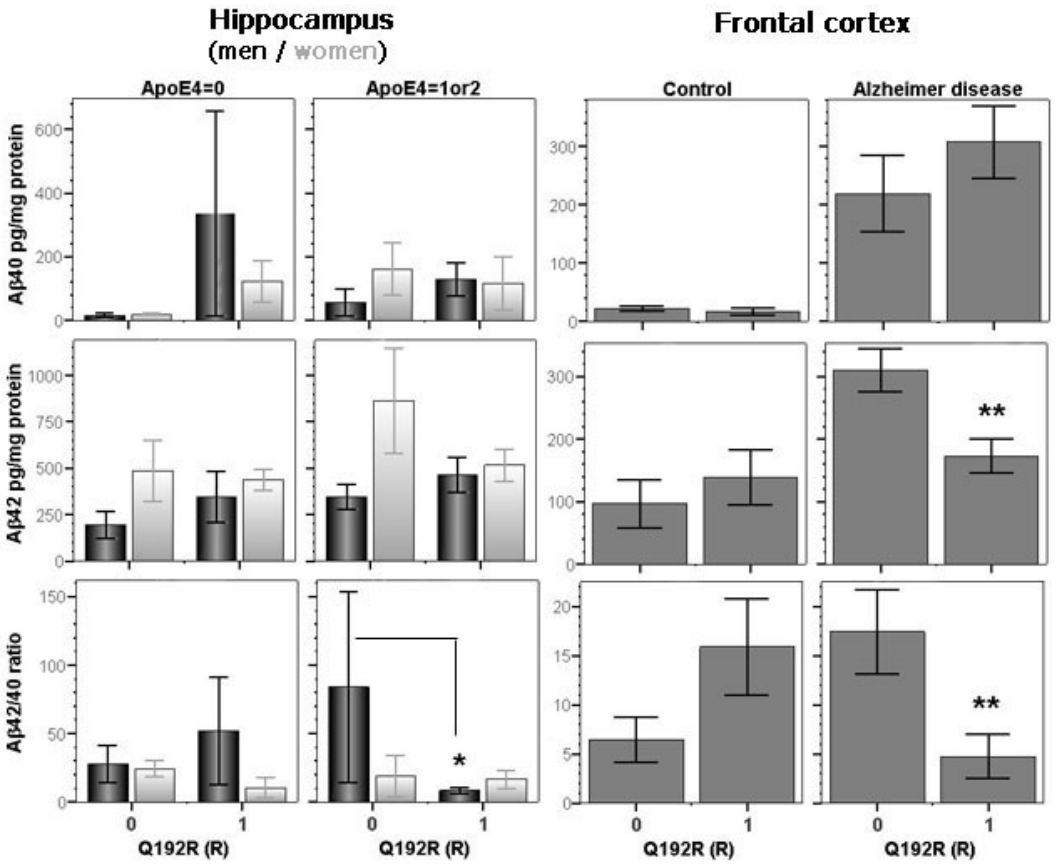

Figure 3.2

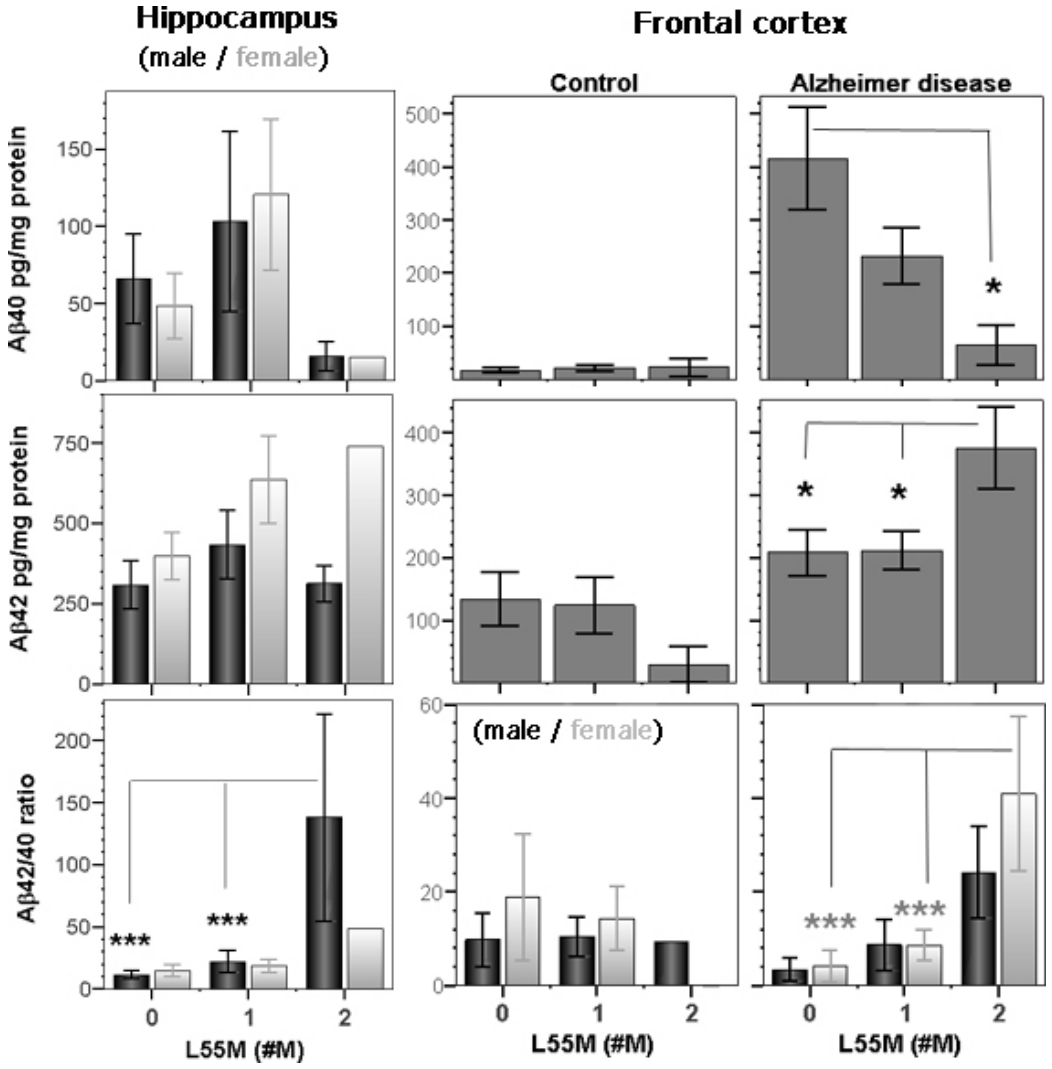
Hippocampus
(male / female)
Frontal cortex
Control
Alzheimer disease
Aβ40 pg/mg protein
Aβ42 pg/mg protein
Aβ42/40 ratio
(male / female)
L55M (#M)
L55M (#M)
L55M (#M)
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Figure 3.3

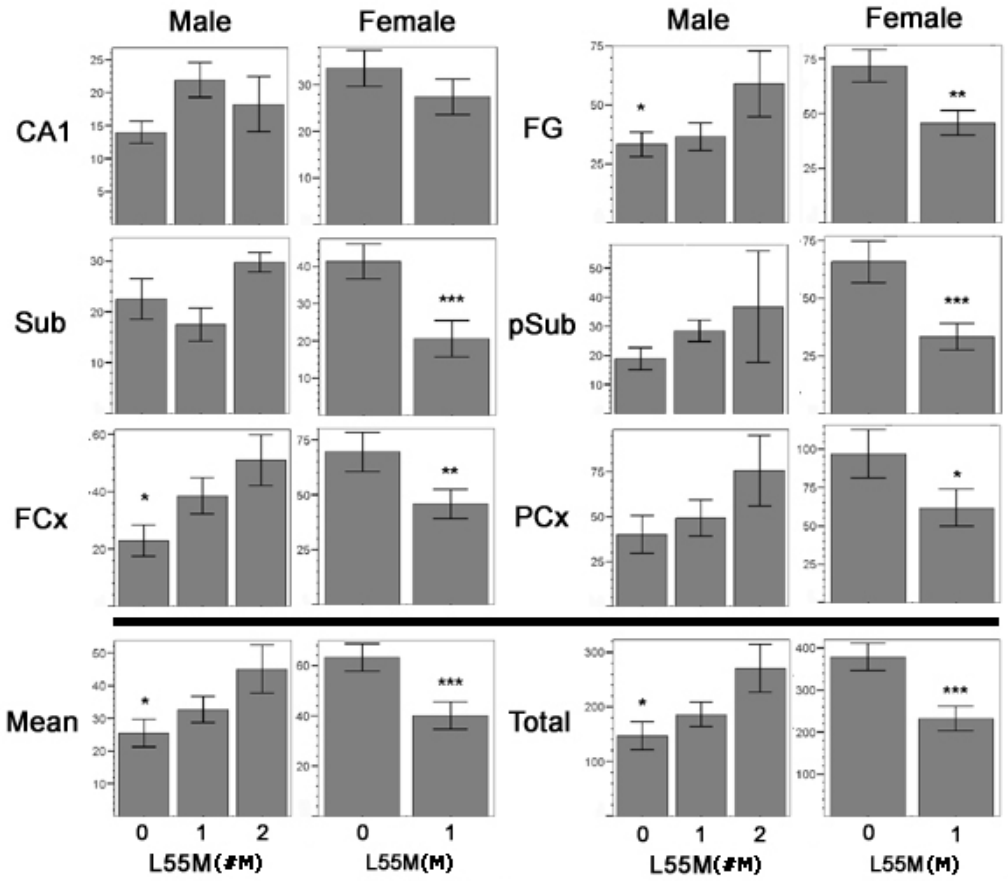
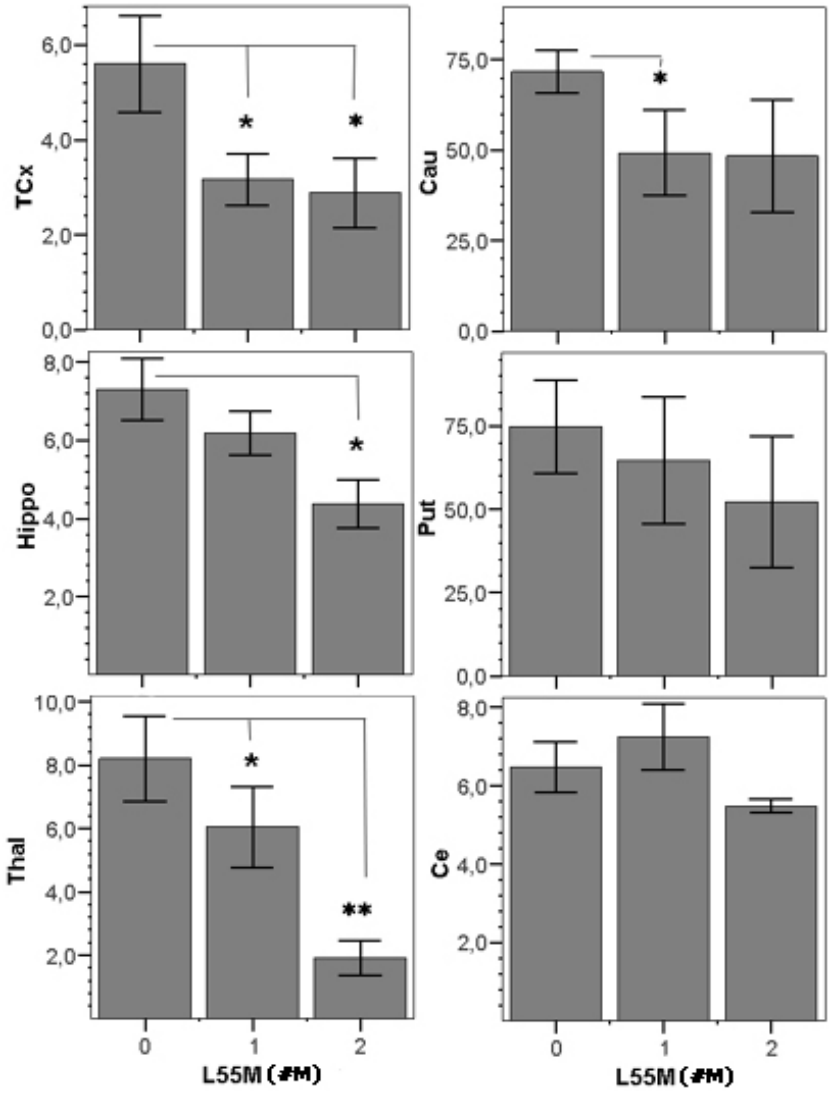

Male
Female
Male
Female
CA1
FG
Sub
pSub
FCx
PCx
Mean
Total
0 1 2
L55M(♯M)
0 1
L55M(M)
0 1 2
L55M(♯M)
0 1
L55M(M)

Figure 3.4



SUPPLEMENTARY MATERIAL

Table 3.1S: Tests for association between PON1 Q192R genetic variants and AD risk (in autopsy-confirmed and clinical cases)

		Overall Effect			Men			Women		
AD	Tests for association	N	p	OR	N	p	OR	N	p	OR
EOAD/LOAD	QQ vs QR	956	0.633	0.937	419	0.824	1.046	537	0.371	0.848
	QQ vs RR		0.519	1.173		0.135	1.783		0.603	0.846
	QQ vs QR/RR		0.822	0.971		0.525	1.132		0.348	0.848
	AT		0.867	1.041		0.258	1.246		0.393	0.900
LOAD	QQ vs QR	885	0.575	0.924	385	0.820	1.050	500	0.320	0.829
	QQ vs RR		0.573	1.155		0.192	1.698		0.640	0.857
	QQ vs QR/RR		0.745	0.957		0.564	1.125		0.313	0.833
	AT		0.952	1.031		0.318	1.224		0.382	0.899

Abbreviations: EOAD, early-onset AD (<65years); LOAD, late-onset AD (≥65years); AT, Armitage's trend test; OR, odds ratio; p, level of significance; Q, glutamine; R, arginine

Table 3.2S: Cox regression analysis of the effect of PON1 Q192R genetic variants on age at death/onset, and AD duration (in autopsy-confirmed cases)

Variable (hazard)	N	Arg status	Exp (B)	p	Conclusion
Age of death (death)	517	0,1	1.167	0.092	Q192R has no effect on age at death/onset or AD duration
Age at AD onset (onset of symptoms)	298	0,1	1.199	0.130	
Duration of AD (death)	298	0,1	0.878	0.271	

Abbreviations: N, number of cases; Arg status (0, 1), Gln/Gln vs Arg/_ genotype; Exp (B), predicted change in the hazard rate; p, level of significance
Disease, apoE4, gender and ethnicity status were included in the analysis as covariates

Table 3.3S: Tests for association between PON1, PON2 and PON3 genetic variants and AD risk (in autopsy-confirmed cases)

SNP nickname	RS#	N cases		HWE		Tests for association (p)
		Control	AD	Control	AD	
PON1						
-162 A/G	705381	90	119	Y	Y	>0.6
Q192R	662	428	528	Y	Y	>0.3
PON2						
Intron 1	2374993	66	111	Y	Y	>0.5
A148G	17354640	107	175	Y	Y	>0.19
Intron 7	12704794	66	112	Y	Y	>0.7
C311S	7493	125	199	Y	Y	>0.9
PON3						
Intron 2	757905	84	148	Y	Y	>0.06
F21F	13226149	87	148	Y	Y	>0.4
Intron 3	6977389	87	147	Y	Y	>0.3
A99A	1053275	89	145	Y	Y	>0.2

Abbreviations: HWE, Hardy-Weinberg equilibrium; Y, yes; p, level of significance

One pound of learning
requires ten pounds of common sense to apply it.

Persian proverb

CHAPTER 4 – ADDITONAL RESULTS AND GENERAL DISCUSSION

Chapter 4. Additional results and general discussion

AD, the most common form of dementia and the fourth leading cause of death in western societies (235), is a complex disorder of heterogeneous etiologies. Many factors may contribute to AD onset and progression and these include, but are not limited to, ageing (95), high cholesterol diet (236), sedentary lifestyle (237), predisposing genetic risk factors (94), dominant inherited mutations (235, 238), and predisposing health conditions such as hypertension, diabetes mellitus and atherosclerotic diseases (239). Factors of greater severity such as dominant inherited mutation in APP gene [e.g. the arctic mutation at codon 693, E693G (238)] may cause EOAD, while cumulative factors of lesser severity such as the ε4 allele in apoE gene (4, 5) may favour LOAD. Although dominant mutations in the APP, PS1 and PS2 genes have been shown to cause EOAD (235, 238), families with such autosomal dominant patterns (a genetic trait located on non-sex chromosome that is pass from one parent to the next generation) represent only 13% of EOAD cases, and about 0.1% of AD cases in general (240-242). In LOAD cases and EOAD cases where the inheritance pattern is unclear, the ε4 allele of apoE gene was identified as a strong genetic risk factor contributing to AD pathogenesis (240-242). The association between ε4 and AD has been robustly confirmed in numerous studies and in several different ethnic groups (reviewed in 94), and accounts for as much as 50% of the susceptibility for AD (108). However, the fact that apoE is neither necessary nor sufficient to elicit AD implies that other causative and susceptibility genes are involved in AD, and have yet to be identified and fully characterized.

One strategy to identify new susceptibility locus is the case-control study (18). Indeed, as proposed by Emahazion and colleagues, association studies can be viewed as useful tools allowing the filtering of large sets of candidate genes in an attempt to identify those potentially playing a functional role in an illness onset and progression (18). Decades of research in the field of neurodegenerative diseases allowed the identification of hundreds of AD susceptibility candidate genes (94). Since apoE lipoprotein complexes are the preponderant lipid carriers

in the CNS, which poorly shares its lipid content with the periphery, we privileged the study of candidate genes involved in lipid and cholesterol transport in our AD case-control analyses. PON1 became an AD susceptibility gene of choice for our studies; its role as a core protective agent of the lipid transport system in the cardiovascular system (19-24, 182, 184-191) represents the perfect candidate to protect apoE lipoprotein complexes in the CNS. Indeed, oxidative stress, a primary event in the course of AD, is extensive in AD brain tissue (79-83, 206-210) and most certainly leads to the formation of oxHDL and oxLDL particles. In turn, this may induce neuronal degeneration (87, 155, 156), and reduce cholesterol transport (157-159), which is crucial for neuronal regenerative and reinnervative processes in the CNS (12, 13, 112, 128, 134, 147). Moreover, the fact that PON1 acts as a key protective agent of the cholinergic system (166, 167, 201), the primary neurotransmitter system affected by AD (67, 73-77, 128), further fuelled our interests in studying this enzyme in the context of AD.

In order to discern real biological signals from false-positive ones, candidate genes identified as a result of association studies should be replicate in a widespread manner (18). Accordingly, numerous PONs' polymorphisms have been identified, and their association with AD, assessed in majority in small heterogeneous populations of the US and Europe, have been controversial. Indeed, some studies demonstrate an association between some PONs' polymorphisms and increased risk to develop AD (1, 25-33), whereas others failed to replicate these findings (34-38). In contrast, we conducted our analyses in a large population of genetically homogeneous Eastern Canadians, and we investigated the effect of PON1 genetic variants on core pathophysiological hallmarks of AD.

We successfully achieved the aims of our study, which were to clarify whether genetic associations exist between PON1 genetic variants and AD on the risk of developing the disease, on its age of onset, on its overall duration and on its neuropathological hallmarks. Among the eleven SNPs that were assessed across the three PON genes, results pertaining to the L55M locus of PON1 gene were especially noteworthy. These results suggest an important gender-specific

involvement of PON1 in AD etiopathology. On the one hand, men carrying a Met allele at the L55M locus of PON1 exhibited: a) an increased susceptibility to develop AD; b) an increased survival and age at onset; c) an increase of both the Aβ42/40 ratio and neuritic SP accumulation in multiple brain areas; and d) a decreased ChAT activity in various brain areas. On the other hand, women carrying a Met allele at the L55M locus of PON1 exhibited: a) an increased survival and age at onset; b) an increase of the Aβ42/40 ratio in both the hippocampus and frontal cortex, ***but*** a decrease in neuritic SP accumulation in multiple brain areas; and c) a decreased ChAT activity in various brain areas. In both sexes, the effect of PON1 L55M genetic variants was mostly dose-dependent, and the presence of two Met alleles was often required in order to obtain a significant association, especially in men.

In both men and women, the Met allele at the PON1 L55M locus appears to be associated with a greater neurotoxic index, given the increased levels of the more neurotoxic form of Aβ peptide (increased Aβ42/40 ratio and Aβ42 levels, Fig.3.2), and the decreased ChAT activity observed throughout the brain (Fig.3.4). Hence, the effect of the Met allele on AD etiopathology is globally similar between men and women and appears detrimental in both cases. However, differences exist between both sexes with respect to PON1 association with neuritic SP accumulation and AD risk (Fig.3.3 and Table 3.2, respectively).

First, sex-related differences in Aβ levels and Aβ deposition were expected in our case/control cohort as such findings have been reported elsewhere (227-229). Females have indeed been repeatedly reported to bear heavier amyloid burden and higher plaque numbers relative to their male counterparts. We did not expect, however, to find a sex-dependent inverse relationship between the Met allele at the PON1 locus and neuritic SP accumulation. The reason for the observed sex difference is unknown at present. Amid the numerous speculations on how sex might interact with the Met allele of PON1 gene to promote neuritic SP accumulation in men, but the contrary in women, one emerged given its simplicity. We found that the neuritic SP counts significantly correlated with the Aβ42 levels in men (both are increased in Met allele carriers), but with the Aβ40

levels in women (both are decreased in Met allele carriers), suggesting that the main Aβ component of neuritic SPs may differ between men and women.

Surprisingly, no clear data exist as to whether the composition of amyloid plaques is the same in men and women or whether it differs. The possible impact of sex is often ignored in reports investigating the composition of amyloid plaques. For instance, a recent paper by Güntert and colleagues demonstrated plaque-dependent differences in Aβ composition between mice and human (46). Using a laser dissection microscopy method to isolate single SP from brain thin section, they confirmed the well-established predominance of Aβ42 in SP of the diffuse type (44, 226), but identified plaque-dependent differences in Aβ composition between two types of neuritic SP, namely cored and compact SPs (see chapter 2, section 2.1.2.1, pages 22-23), the former being mainly composed of Aβ42 and the latter of Aβ40 (46). In their sample, human AD patients (5cases, sex not specified) and PS2/APP mice (3cases, sex not specified) showed clear differences in the occurrence of plaque types, human having 90% cored / 10% compact SPs and PS2/APP mice having 10% cored / 90% compact SPs (46). Many factors have been proposed to account for the specie's differences (46), but surprisingly, the impact of sex within as well as between species was not investigated. These findings, combined with both our gender-specific neuritic SP accumulation data and with the increase SP deposition reported in female relative to male (227-229), raise the question of whether profound differences could exist between sexes with respect to SP formation, composition and maturation. Although we accumulated no biochemical evidences to support such an hypothesis, we hope our results will prompt other research teams to investigate this matter.

Second, while the reason for the observed sex difference between the Met allele at the PON1 locus and AD risk is unknown at present, similar sex-specific associations with AD have been reported for other genes (224-225). Accordingly, research in the past 10 years has increasingly documented sex influences on brain anatomy, function and chemistry (reviewed in 216). For instance, male and female hippocampi differ considerably in their anatomical structure,

neurochemical make-up and reactivity to stressful situation (216, 243). Quite interestingly, chronic stress was shown to cause damage to the hippocampus of rat and monkey males, but not of females (216, 244). This implies that female hippocampal cells are far more resistant than male hippocampal cells to stress-induced damage (216). While the exact mechanisms mediating the increased resistance of female hippocampal cells to chronic stress remain elusive, male sex hormones have been shown to impair immune function and wound healing following injuries, and to decrease the activity of antioxidant enzymes such as superoxide dismutase and catalase (245, 246). We proposed that the detrimental impact of male sex hormones on these three biological processes, which are extensively called upon during neurodegeneration, might contribute to increase the vulnerability of males to stresses. If polymorphisms decreasing the protein and activity levels of antioxidant enzymes such as PON1 are present, they may further increase the vulnerability of males in stress-generating contexts such as aging and AD. Although speculative, this might contribute to explain, in part, why the PON1 Met allele, which is associated with decreased PON1 protein and activity levels (167, 171, 218-221), promotes AD and neuritic SP accumulation in men, but not in women.

Although the impact of gender on the activity of antioxidant systems remains to be clearly established, two sets of studies suggest a gender dimorphic regulation of PON1. First, Bin Ali and colleagues demonstrated that PON1 mRNA expression in rodents was gender-dependent: while ovariectomy had no effect in females, castration increased hepatic PON1 mRNA in male by 170% to a level comparable to that of females (247). Second, women suffering from polycystic ovary syndrome, a disease characterized by increased ovarian and adrenal *androgen* secretion (248), were shown to exhibit decreased PON1 levels relative to normal healthy women (248, 249). Because a significant decrease of PON1 activity in males relative to females would be consistent with our observed enhanced AD risk and neuritic SP accumulation in men, we wondered whether the gender dimorphic regulation of PON1 mRNA seen in rodents could also exist in humans. Hence, we quantified the mRNA levels of PON1 in the frontal cortex of

AD and age-matched non-demented control subjects using real-time reverse transcriptase-polymerase chain reaction (real time RT-PCR) (see appendix 1).

Our mRNA data did not support a gender dimorphic regulation of PON1 mRNA, as these mRNA levels were not significantly different between men and women in either our control or AD cohort ($p>0.05$). This could be indicative, among other possibilities, of a gender dimorphic regulation of PON1 mostly mediated by gender-specific post-translational modifications or gender-dependent degradation of PON1 proteins in humans. Thus, the mechanisms by which sex might interact with PON1 Met allele to promote AD and increased neuritic SP accumulation in men, but decreased neuritic SP accumulation in women, remain elusive. The impact of sex on the associations between PON1 and both AD risk and neuritic SP accumulation clearly needs to be further investigated.

As discussed in chapter 3, the evidences we gathered suggest a functional involvement of the coding L55M SNP in AD. However, given the strong impact of Q192R genetic variants on Aβ levels (Fig.3.1), the Met allele at the L55M locus most probably acts in concert with other nearby PONs' polymorphisms to influence AD etiopathology. This hypothesis is supported by two observations.

First, genetic variants at the PON2 (see chapter 2, section 2.3.1, page 44) locus have been shown to considerably affect, independently of PON1 genetic variants, ChAT activity in the hippocampus of AD and non-demented control subjects (see appendix 2, Fig.A.2). The possible involvement of PON2 in AD is further supported by the observation that PON2 mRNA levels are upregulated by 62% in the frontal cortex of AD relative to age-matched control subjects ($p<0.001$, Table A.1, appendix 1). Considering PON2 intracellular antioxidant function in the periphery (180), it could very well represent the perfect complement to PON1 extracellular antioxidant function. We are currently investigating if PON2 genetic variants or mRNA levels correlate with the intracellular-generated NFTs, the only set of neuropathological data that was not associated with PON1 genetic variants.

The increased survival and age at onset associated with the Met allele constitute the second set of observations supporting the hypothesis that PON1 Met

allele may act in concert with other PON1 polymorphisms or other genes to influence AD etiopathology. While the mechanism by which the Met allele is associated with a later age of onset of AD is unclear, the increased survival observed for MM carriers corroborates the results of studies performed on centenarians, which suggested a role for the 55Met allele in the extended longevity phenotype (250-252). Interestingly, this counterintuitive increase of originally harmful alleles (the Met allele is associated with an increased risk for vascular diseases [221] and AD [1, 25-33]) in centenarians has been reported for other risk factors (see 253 for references), and is easier to understand if the biological and physiological roles of the respective alleles in survival are considered (253). Because the physiological functions of PON1 remain to be clarify, it is difficult to explain why an individual carrying a potentially harmful Met allele, who yet survived under the pressure of selection and reached very old age, may get an advantage from the same allele much later in life (253). However, an *in vitro* study performed in the late 90s by Mackness and coworkers paves the way for a hypothesis (254). Although the Met allele has been associated with decreased PON1 protein and activity levels (166, 167, 218-220), Mackness et al demonstrated that HDLs from 55MM/192QQ individuals were more efficient at protecting LDL particles from oxidation than HDLs from 55LL/192RR individuals (254). As such, a 55MM/192QQ carrier possessing a strong antioxidant machinery might be more inclined to become centenarian, whereas he/she might be prone to develop stress-related diseases if his/her other antioxidant defence systems are weaker. These latter carriers will be especially prone to develop age-related diseases such as vascular diseases and AD, as oxidative stress increases with age (95).

Everything should be made as simple as possible,
but not simpler.

Albert Einstein

CHAPTER 5 – CONCLUSION

Chapter 5. Conclusion

In sum, no biochemical evidence has been accumulated so far to support an irrefutable hypothesis on how the L55M coding polymorphism might promote AD and influence its neuropathology. The most plausible hypothesis is that the Met allele might influence the clearance of Aβ peptides. Given the fact that Aβ peptides are normally found attached to lipoprotein particles upon secretion by cells (104, 123-127), lipoprotein particles have been proposed to act as scavengers of Aβ peptides (106). As aforementioned in chapter 3, if the Met allele is indeed associated with decreased levels of PON1 protein and activity, it could promote the generation of oxHDL whose electrical charges will repulse lipophilic Aβ peptides (Fig.5.1). This could result in decreased HDL scavenging function, followed by local increase in Aβ peptide concentrations, and eventually, in the formation of neurotoxic prefibrillar species of Aβ, which could compromise the neuronal integrity of highly metabolically active neurons such as the cholinergic neurons (1) (Fig.5.1). To support this hypothesis, we attempted to quantify the mRNA, protein and activity levels of PON1 in a case-control cohort of 90 patients. However, only the mRNA levels were successfully quantified. Our real time RT-PCR data indicated that PON1 mRNA levels are upregulated by 32% in the frontal cortex of AD relative to age-matched control subjects ($p<0.05$, Table A.1, appendix 1). Additionally, in the control subgroup, $apoE4^+$ individuals carrying at least one Met allele had lower PON1 mRNA levels relative to $apoE4^+$ LL carriers (78% less, $p<0.05$, Table A.1, appendix 1). The effect of the Met allele on PON1 mRNA levels in $apoE4^+$ control subjects was, however, not observed in $apoE4^+$ individuals affected by AD ($p>0.05$). Because the protein and activity levels of PON1 in our case-control cohort are so far unavailable, the mechanisms by which the Met allele is associated with lower mRNA levels in $apoE4^+$ control subjects remain elusive. Nevertheless, the observed effect of the Met allele on PON1 mRNA levels in $apoE4^+$ control subjects provides evidence supporting a functional involvement of the coding L55M SNP in PON1 expression.

Although we weren't able to quantify the brain protein levels of PON1, the Swedish Human Protein Atlas program has demonstrated the presence of PON1 protein in the cortex and cerebellum of non-neoplastic and morphologically normal human subjects (freely available at http://proteinatlas.org/tissue_profile.php?antibody_id=1610). Our protein quantification analysis probably failed because of a lack of specificity from our PON1 antibody rather than a lack of presence of PON1 protein in the human brain. The presence of PON1 protein in the human brain is further supported by the detection of arylesterase activity in the frontal cortex of our case-control cohort (unpublished preliminary data, see appendix 3). PON1 protein and activity levels will be the subjects of future studies in our laboratory.

In conclusion, it has been suggested that apoE lipoprotein particles may be involved in synaptic plasticity during regeneration and repair (134), and the genetic data we have gathered here suggest that the Met allele of PON1 might impair this function by promoting the oxidation of lipoproteins (1) (Fig.5.1). However, biochemical analyses are needed to support this hypothesis, notably the quantification of brain PON1 protein and activity levels in our case-control cohort. Additionally, it would be interesting to see if PON1 knockout mice, which exhibit an increased sensitivity to the toxicity of OP and increased susceptibility to develop atherosclerosis (19-21), exhibit an age-dependent decrease in synaptic density and Aβ peptide deposition. It would also be interesting to see if neural tissue culture cells demonstrate decreased neurite outgrowth in the presence of PON1-deficient lipoproteins relative to wild-type lipoproteins. Nevertheless, our results further emphasize the importance of lipid homeostasis and oxidative stress in SAD, as well as the need to further study PON1 function in the human brain. Additionally, our results stress the importance of investigating the effects of sex in neurodegenerative disorders since we found intriguing sex-dependent inverse relationships between AD etiopathology and PON1 gene.

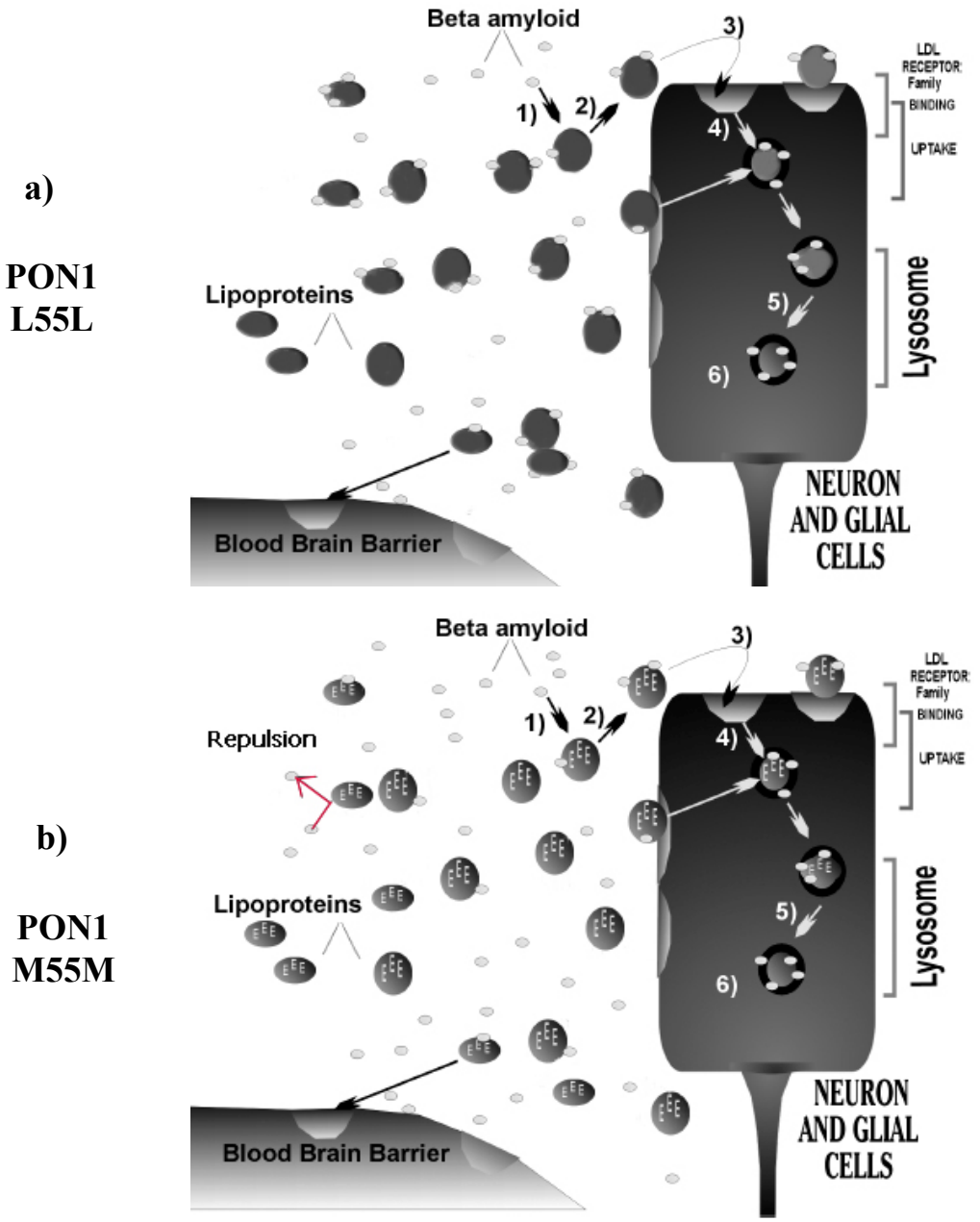


Figure 5.1: Schematic representation of postulated scavenging function of apoE lipoproteins containing Leu or Met alleles at residue 55 of PON1 in the mature brain. In this model, apoE lipoproteins initially bind to the lipophilic portion of soluble Aβ peptides (1). The resulting complex next binds to cell surface receptors belonging to the LDL receptor family (2 and 3). The receptor/ligand complex is then internalized via a clatherin-coated pit-mediated process (4), and then directed to the endosomal compartment (5), where it is processed through the usual endosomal/lysosomal pathway (6). ApoE lipoprotein complexes containing *a)* L55L PON1 or *b)* M55M PON1. The Met allele is postulated to be associated with decreased PON1 protein and activity levels, which could promote the generation of oxidized HDL whose electrical charges (E) will repulse lipophilic Aβ peptides, and thus prevent its degradation. Pictures adapted from (106).

One never notices what has been done;
one can only see what remains to be done.

Marie Curie

APPENDICES

Appendix 1. PON1 and PON2 mRNA levels

Table A.1: PON1 and 2 mRNA levels in the frontal cortex of control and AD subjects:

	Cohort		Control subgroup		AD subgroup	
	Control	AD	apoE4$^-$	ApoE4$^+$	apoE4$^-$	ApoE4$^+$
Number of cases [a]	36	54	22	14	19	35
Sex (M/F)	23/13	25/29	12/10	11/3	8/11	17/18
PON1 mRNA	ns	Upregulated 32.4%*	-	-	-	-
PON2 mRNA	ns	Upregulated 61.8%***	- -	- -	-	-
L55M						
LL carriers	ns	ns	-	-	-	-
ML+MM carriers	ns	ns	Downregulated 78% [#]	ns	ns	ns

Data were obtained by pair wise fixed reallocation randomization test, and were normalized against UBE2D2, ubiquitin-conjugate enzyme E2D2. ns, non significant; apoE4$^-$, carry no E4 allele; apoE4$^+$, carry at least 1 E4 allele.

[a] Age at death and PMD are not significantly different between groups

* Different from control $P<0.05$

*** Different from control $P<0.001$

[#] Different from LL carriers $P<0.05$

Method:

PON1 and PON2 mRNA levels were quantified relatively to an endogenous reference gene (ERG). Briefly, RNA was extracted from human post-mortem frontal cortex tissues using QIAGEN RNeasy kit for lipid-rich tissue; specific primers allowed PON1 and the ERG mRNA to be reverse transcribed into cDNA (RT step); in turn, these cDNA were amplified by PCR (PCR step) with the SybrGreen method (with dissociation protocol) using a Gene-Amp 5700 Sequence detection system from PE Applied Biosystem; and finally, results were expressed as PON1/ERG ratios and analyzed using REST-XL software (255) by means of pair wise fixed reallocation randomization test.

The strength of our real-time RT-PCR analyses resides in the fact that we carefully tested and validated the expression stability of the ERG in our case-control cohort. We assessed 10 potential housekeeping genes (selected from literature) on 9 patients of different gender, disease status (control or AD), ethnicity, and apoE genotype, and selected the most stable one using the BestKeeper, NormFinder, and geNorm softwares (256-258). The ERG selected was ubiquitin-conjugate enzyme E2D2 (UBE2D2).

Appendix 2. PON2 and ChAT activity

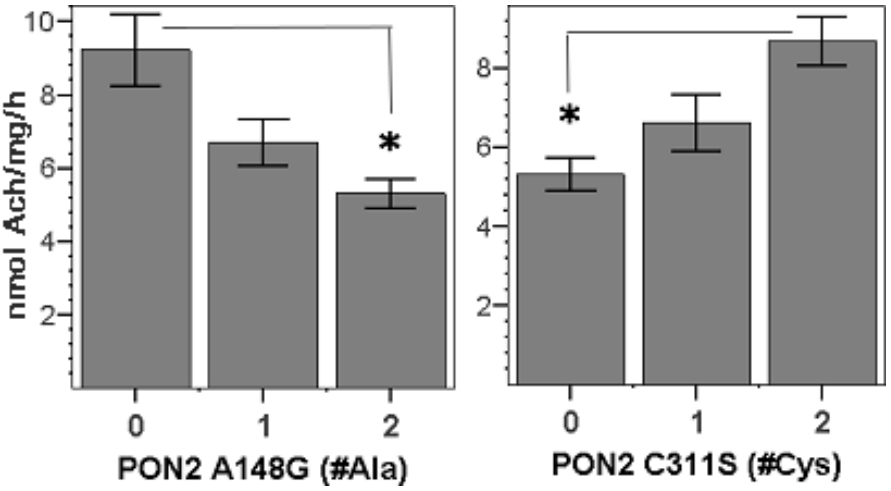


Figure A.2: Effect of PON2 A148G and C311S variants on ChAT activity in the hippocampus (N=43) of autopsy-confirmed AD and age-matched control cases. Data are expressed as nmol Acetylcholine/mg protein/h ± SEM, and contrast carriers of 0, 1 or 2 Ala and Cys alleles for respectively A148G and C311S polymorphisms. Mean PMD varies between 20 and 30hrs and has no significant impact on ChAT activity. Disease status, gender, ethnicity status, apoE4 presence and PON1 L55M genetic variants were included in the analysis as cofactors. Significantly different * $p<0.05$, (ANOVA, Bonferroni's Post-Hoc). Note that A148G and C311S polymorphisms are in high linkage desequilibrium towards one another.

Method:

See "ChAT activity" in the material and methods section of chapter 3.

Appendix 3. PON1 arylesterase activity

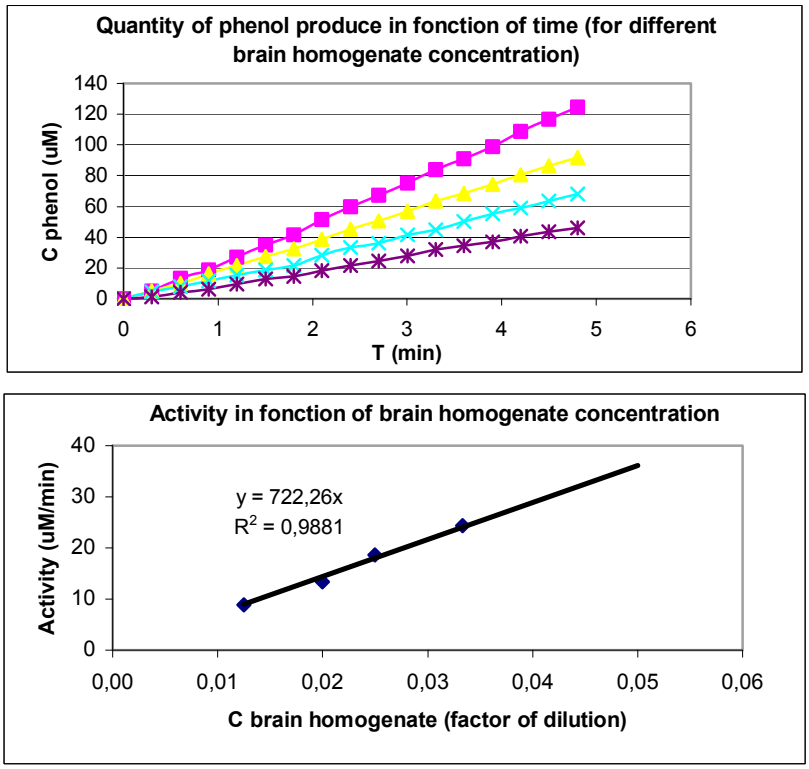


Figure A.3: Hydrolysis of phenylacetate by the frontal cortex homogenate of an AD subject (arylesterase activity of PON1). C, concentration; T, time.

Method:

Frontal cortex slices were homogenized in 10 volumes of 50mM Tris–HCl (pH 8.0) containing 2 mM of phenylmethyl sulfonyl fluoride (PMSF) to inhibit serine esterases, and the homogenates were centrifuged at 10,000 × g for 15min. Then, supernatants were incubated for 30min on ice in the presence of 10μM paraoxon to inhibit B-type esterases, which could interfere with PON1 arylesterase assay. Enzyme activity toward phenylacetate was determined by measuring the initial rate of substrate hydrolysis in the assay mixture containing 3mM substrate, 2mM $CaCl_2$, and 1M NaCl in 100mM Tris–HCl (pH 8.0). The absorbance was monitored for 20min at 270nm, and the activity was calculated from the molar extinction coefficient $E_{270} = 1310\ M^{-1}\ cm^{-1}$.

Appendix 4. Research compliance certificates

Centre McGill d'études sur le vieillissement

McGill Centre for Studies in Aging

March 29, 2007

Dr. Judes Poirier
Senior Scientist
Douglas Hospital Research Centre
and
Mrs. Danielle Cécyre
Coordinator
Douglas Hospital Brain Bank

RE: Use of the Brains and DNA obtained from the Signalgene Corporation

This latter is to confirm that Dr. Judes Poirier is authorized by the Research Ethic Board to fully access and use the blood, DNA and brain tissues of the subjects obtained as part of the SignalGene collection to perform research studies on the causes and treatment of Alzheimer's disease as per protocol 06/19.

However, only the tissues from subjects carrying an Identification Number starting with the prefix SA are to be accessed for such studies. The families of these specific cases were re-contacted by SignalGene in the 1997-1999 period and the informed consents were reconfirmed (and documented) for research projects dealing with the genetic causes and treatments of Alzheimer's disease.

Since Dr. Poirier's research program on the etiology and genetics of Alzheimer's disease falls directly within the original intent of the program, he is authorized to perform this research using the most appropriate technology.

Finally, it should be clear that the name of the subjects will be kept confidential and that only the Identification Numbers will be used to identify the subject's demographic characteristics and their relations to the genetic markers.

Sincerely,

Dr. Serge Gauthier
Président du Comité d'éthique
Cc: Lise Bourgon

Centre McGill d'études sur le vieillissement • McGill Centre for Studies in Aging
Hôpital Douglas Hospital
6825, boul. LaSalle Blvd., Montréal (Verdun) Québec, Canada
Tél.: (514) 766-2010 • Téléc./ Fax: (514) 888-4050
Courriel / E-Mail: info.mcsa@mcgill.ca

Ouverts d'esprit. Depuis 125 ans
Caring with Open Minds. For 125 Years

Hôpital Douglas Hospital

December 15, 2006

Dr. Judes Poirier
Douglas Hospital Research Centre
McGill Center for Studies in Aging
6825, LaSalle Blvd.
Verdun, Quebec
H4H 1R3

Subject: **Protocol 02/34** *Identification of Novel Genetic Risk Factors in Autopsy-confirmed Sporadic Alzheimer's Disease*
Annual Renewal

Dear Dr. Poirier;

Thank you for the annual report you submitted for approval for the above protocol. As Acting Chairperson, I have examined your report and found it satisfactory. I therefore give expedited approval to this annual renewal request since it is complete and it meets REB requirements.

This study is re-approved for a one-year period.

Sincerely yours,

for:
Jacques-Bruno Debruille, M.D.
Acting Chairperson
Douglas Hospital Research Ethics Board
/lb

January 18, 2008

Dr. Judes Poirier
Douglas Institute Research Centre
McGill Centre on Aging
6825, LaSalle Blvd.
Verdun, Quebec
H4H 1R3

Subject: Protocol 02/34 *Genetics of Sporadic Alzheimer's Disease*
- **Annual Renewal**

Dear Dr. Poirier;

Thank you for the annual report you submitted for approval for the above protocol. As Acting Chairperson, I have examined your report and found it satisfactory. I therefore give expedited approval to this annual renewal request since it is complete and it meets REB requirements.

This study is re-approved for a one-year period.

Sincerely yours,

for :
J. Bruno Debruille, M.D., Ph.D.
Acting Chairperson
Douglas Institute Research Ethics Board
/lb

Appendix 5. Signed waiver

Date: Thu, 24 Jul 2008 14:03:22 -0500 [07/24/2008 03:03:22 PM EDT]
From: Brand Help <brandhelp@alz.org>
To: valerie.leduc@mail.mcgill.ca
Subject: RE: Request permission

Hello,

Thank you for your interest in generating greater awareness about Alzheimer's.

You have our permission to reproduce the entire brain tour as a compilation for non-commercial usage. Please include the following:

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Brandhelp@alz.org

-----Original Message-----
From: valerie.leduc@mail.mcgill.ca [mailto:valerie.leduc@mail.mcgill.ca]
Sent: Wednesday, July 23, 2008 10:48 AM
To: Brand Help
Subject: Request permission
Importance: High

Good day,

I am requesting permission to reproduce the following image in my master thesis at McGill University (Montreal, Quebec, Canada):

Inside the Brain: An Interactive Tour. Slide 9 Available at:
http://www.alz.org/brain/overview.asp Accessed July 23, 2008

The thesis will not be used for commercial purposes.

Thank you for your help and consideration.

Sincerely,

Valérie Leduc
MSc candidate

Cunning is the dwarf of wisdom

William R. Alger

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