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Douglass J. Stennett

Recent in vitro data provide evidence that estrogen may assist in the maintainance of nervous tissue viability. Anecdotal reports suggest a lower prevalence of SDAT in postmenopausal women who have received estrogen replacement therapy although published data are conflicting. The purposes of this study are to determine whether the incidence of SDAT differs between males and postmenopausal females, and whether an inverse association exists between postmenopausal estrogen replacement therapy and SDAT. The data sets analyzed were the Oregon Public Employees Retirement System (PERS) (n=18,893) and pharmacy databases for 118 long term care facilities in Oregon (n=3141). Logit analysis odds ratio, adjusted for age, for conjugated estrogen exposure was 0.51 (95% confidence interval 0.26 - 0.97). Although daily dose and length of therapy also indicated a reduced odds ratio for estrogen users vs. non-users, the results are equivocal with this database. In conclusion, this database provides evidence that estrogen replacement therapy has a positive impact on the prevalence of SDAT in postmenopausal women, although the effect of length of treatment and total cumulative dose remain unclear.

Prevalance of Senile Dementia of the Alzheimer Type (SDAT) in Females Receiving Postmenopausal Conjugated Estrogen vs. Controls; A Retrospective Review

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Michael D. Gooch

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Introduction

The question of why people develop Alzheimer's disease has gained importance and urgency in recent years as the average life span has increased from 60 years at the turn of the twentieth century to 85 years today. Alzheimer's disease was first described in 1907, at a time when persons over the age of 65 years represented only 3% of the United States population. It is estimated that by the year 2020 that percentage will have increased to include one-fifth of the U.S. population, some 55 million people.[1] Alzheimer's disease is the most common dementia of the elderly, comprising 50% of the cases of diagnosed dementia.[2] This is followed by multi-infarct dementia (20-25%), alcoholic dementia, Parkinsonian dementia, hypoxic encephalopathy, psychosis, and post-traumatic encephalopathy. It is clear the incidence of Alzheimer's disease increases with age, affecting approximately 10-13% of individuals over the age of 65 years but up to 35-48% of those over 80 years.[3,4] There is currently no effective treatment, and certain diagnosis depends upon obtaining a brain biopsy from the patient, a procedure rarely opted for due to its obvious risks.

Evidence exists to suggest that declining estrogen levels following the onset of menopause may be a risk factor for the development of Alzheimer's disease in females. Some studies have indicated the incidence of Alzheimer's disease, after adjusting for age, is higher in females than in males.[5,6] Males possess an enzyme in the brain called aromatase which can convert testosterone into estrogen,[7] and aromatase may serve as a protective factor in determining a male-female prevalence difference based on estrogen deficiency. Estrogen levels have been reported to be lower in post-menopausal females than in males the same age. Also estrogen supplementation in post-menopausal women with Alzheimer's disease has been shown to be only one-third that of age-matched controls who were unaffected.[8] Identification of protective factors which may render the aging female less likely to develop the disease or delay its progression would be useful. Female menopause normally occurs between the ages of 42 and 60 years, but changes in endocrine function are not abrupt. Serum concentrations of estrogens, estrone and estradiol, decline from average levels of 0.08 ± 0.01 and 0.05 ± 0.005 ng/mL to levels of

0.029±0.002 and 0.013±0.001 ng/mL respectively following menopause.[9] Estrogen may protect against Alzheimer's disease in a number of ways. It may alter the metabolism of the amyloid precursor protein, a cell surface protein from which β-amyloid, the primary constituent of the plaques found in the brains of Alzheimer's disease patients, is formed.[10] Diverting amyloid precursor protein metabolism toward non-amyloidogenic pathways, may slow or prevent the formation of senile plaques. Estrogen may also modulate neuronal response to injury via effects on nerve growth factors and their receptors.[11] Finally estrogen may prevent the decline in cholinergic transmission thought to be behind the memory deficits reported in Alzheimer's disease patients. Estrogen administration in animals has been shown to increase the mRNA for choline acetyltransferase (ChAT), an enzyme important for production of the neurotransmitter acetylcholine.[12] ChAT has been reported to be deficient in the basal forebrains of Alzheimer's patients as compared to controls.[13]

Estrogen replacement therapy is commonly prescribed in post-menopausal women to relieve the discomfort of vasomotor symptoms associated with the onset of menopause and to prevent the development of bone loss which could lead to an increased risk of fractures. In 1994 one brand of conjugated estrogen, Premarin^{R}, was the most frequently prescribed medication in the United States.[14] The goals of this study are to determine whether the prevalance rates of Alzheimer's disease differs between males and post-menopausal females when adjusted for age, and whether an inverse association exists between Alzheimer's disease and post-menopausal estrogen use. This will be accomplished by examining data for both members of the Oregon Public Employees Retirement System (PERS) regarding prescription drug use and office visits to physicians for Alzheimer's disease, and a survey of over 3000 patients residing in long-term care facilities within the state of Oregon. If an inverse association is found to exist between estrogen use and the development of Alzheimer's disease, is it affected by age, by dose, and is it altered by concominant use of other medications commonly prescribed for the elderly?

Background

Diagnosis

A definitive diagnosis of Alzheimer's disease can only be made based on a neuropathological examination at autopsy. Until the diagnosis can be confirmed at death the patient is considered to have Senile Dementia of the Alzheimer's type (SDAT). Accuracy of diagnosis has improved considerably over the last decade. Until recently SDAT has been considered a diagnosis of exclusion. The development of inclusion criteria for the disease has been an important development. Use of standardized neuropsychological testing in conjunction with a thorough medical examination can give an accuracy of diagnosis of greater than 90%.[15,16] Neuroimaging studies are of value primarily to rule out other causes of dementia such as neoplasms or infarcts. Unfortunately no consistently accurate markers can be identified from tests such as computerized axial tomography (CT).[17] Neuropsychological tests can distinguish dementia from normal aging but do not differentiate well among the different types of dementia. This is due to an incomplete knowledge of the neuropsychological profile of some of the less common types of dementia, considerable overlap among the cognitive deficits observed among the different dementias, and the difficulty in differentiating Alzheimer's disease symptoms from those of depression. There are certain deficits that should place the diagnosis of Alzheimer's in doubt. These include focal signs, motor impairment, severe attention deficit, or predominant speech problems early in the disease.[18] The National Institute of Neurological and Communicative Disorders/ Stroke-Alzheimer's and Related Disorders Association (NINCDS/ADRDA) Work Group criteria for clinical diagnosis of probable Alzheimer's disease are outlined in Table 1.[19]

Table 1: NINCDS/ADRDA standardization of criteria for diagnosis

1. **Dementia established by clinical examination & documented by objective testing (Mini-Mental State Exam, CERAD).**
2. **Deficits in two or more cognitive areas.**
3. **Progressive worsening of memory or other cognitive functions.**
4. **No disturbance in consciousness.**
5. **Onset between the ages of 40 and 90.**
6. **Absence of systemic disorders or other brain diseases which could account for the progressive deficits.**

Pathologically, Alzheimer's disease is characterized by the presence in the brain of senile plaques, neurofibrillary tangles, and loss of cholinergic neurons of the basal forebrain. Deposits of amyloid, the primary protein found in senile plaques, can also be detected in the walls of cerebral blood vessels. The relationship between these different lesions is a matter of much debate.

β-amyloid and Amyloid Precursor Protein

Senile plaques found in the brains of Alzheimer's disease patients consist of a central core of radiating amyloid fibrils surrounded by dystrophic neurites. Amyloid plaques are sometimes seen in the brains of non-demented individuals but are much more extensive in Alzheimer's disease. At the ultrastructural level amyloid deposits consist of unbranching fibrils 6-10nm in diameter. The histological diagnosis of amyloid is based almost entirely on its staining characteristics. The demonstration of a green to yellow birefringence under polarized light after staining with the dye congo red is due to the β-pleated sheet configuration of the amyloid. Other techniques utilize fluorescence after staining with thioflavin T and S.[20] A second type of amyloid deposit called diffuse amyloid plaques consists of an amorphous, cotton wool-like deposit of amyloid. Diffuse plaques do not stain with congo red and are not surrounded with dystrophic cells.[21] They may be precursors to the senile plaques. In a study of 15 non-demented centenarians, 95% of the amyloid deposits found at autopsy were of the diffuse type.[22] Diffuse plaques may develop as a normal process of aging.

The main component of cerebrovascular amyloid, a 39-42 amino-acid polypeptide, was purified and sequenced by Glenner and Wong in 1984.[23] Subsequent research showed it to be identical to amyloid isolated from senile plaques. This 39-42 amino acid segment will be referred to here as β-amyloid although βA4 and Aβ are also frequently used abbreviations in the literature. β-amyloid is derived from a 695-770 amino acid precursor, called the amyloid precursor protein (APP)[24] (Figure 1).

Numerous observations support a role for β-amyloid in the pathogenesis of Alzheimer's disease. The gene for APP is located on chromosome 21 and individuals with Down's Syndrome (trisomy 21) almost invariably develop Alzheimer's disease if they survive into middle age, presumably due to the extra gene-dose of APP they acquire.[25] Down's Syndrome carries with it a significant risk for early mortality due to an increased incidence of leukemia and congenital heart disease, therefore an extensive autopsy record exists for this group of individuals. Diffuse plaques have been reported to be present in the

early teens, followed by development of increasing numbers of senile plaques, which precede the development of neurofibrillary tangles. [26]

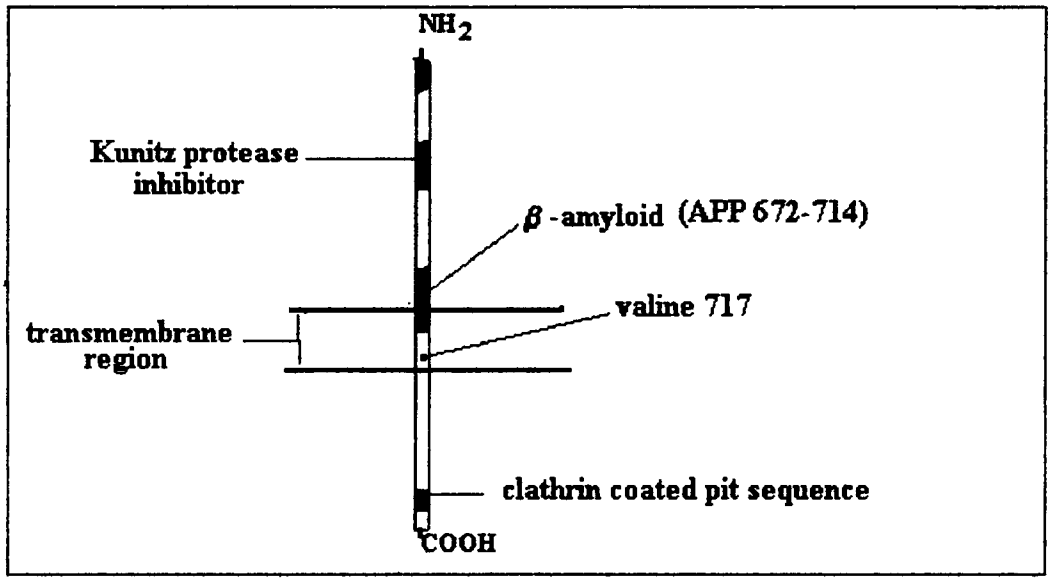

Figure 1: Amyloid precursor protein

Familial early-onset forms of Alzheimer's disease co-segregate with mutations of the APP. A mutation of valine 717 (using APP_{770} numbering) to either isoleucine, phenylalanine, or glycine is found in these families. The location of the missense substitution is outside the β-amyloid region suggesting the mutation alters the proteolytic processing of APP toward amyloidogenic pathways. In support of this theory transfected neuroblastoma cell lines expressing APP_{717} mutants express more of the long forms (1-42 amino acids) of β-amyloid which form insoluble amyloid fibrils more rapidly than the 1-40 forms.[27] Recently a transgenic mouse expressing the APP_{717} mutation (phenylalanine for valine) has been produced. These mice progressively develop many of the characteristic pathologies of Alzheimer's disease, including diffuse and senile plaques, but not neurofibrillary tangles.[28] A second early-onset mutation is a double substitution changing the lysine and methionine located at positions 670 and 671 (again APP_{770} numbering) to asparagine and leucine. These mutations are also outside of the β-amyloid region suggesting an alteration in metabolism. This is also supported by cell culture studies using neuroblastoma cell lines. Cells transfected to express the double mutation form of APP

secrete five-fold more β-amyloid into the cell culture medium than do control cells expressing the wild-type APP.[29]

Another mutation of APP, this time at position 693 of APP_{770} located within the β-amyloid sequence, substitutes a glutamine for a glutamate. This substitution is found not in patients with early-onset Alzheimer's disease but in Dutch Cerebral Amyloid Angiopathy. This single amino acid substitution alters the secondary structure of β-amyloid resulting in increased aggregation.[30] Patients with this syndrome develop deposits of β-amyloid in the blood vessels of the brain leading to massive strokes. No neurofibrillary tangles are seen.

Numerous cell culture models have demonstrated the neurotoxicity of β-amyloid.[31,32] After exposure to β-amyloid, hippocampal cell cultures demonstrate increased vulnerability to excitotoxic amino acids.[33] The length and configuration of the β-amyloid fragment appears to be an important factor in its toxicity. Longer lengths (1-42 versus 1-40) and a fibrillar conformation show increased aggregation and neurotoxicity.[34] Amino acid residues 1-8 of the polypeptide are predicted to have an α-helix structure. Residues 9-13 and residues 22-28 are predicted β-turns. The rest of the peptide has a predicted β-sheet structure.[35]

APP exists as multiple isoforms from a single gene located on chromosome 21. The most common isoforms lengths being designated as APP_{695}, APP_{751}, and APP_{770}. APP_{695} is the most common isoform in human brain. However, APP_{770} has been isolated from regions of the brain affected by Alzheimer's disease.[36] APP contains a single membrane spanning region, an amino terminal extracellular region comprising approximately two-thirds of the length of the peptide, and a carboxy terminal cytoplasmic region. The β-amyloid fragment begins 28 amino acids on the amino side prior to the start of the transmembrane domain of APP and concludes with the first 11-14 amino acids inside the membrane. APP is ubiquitously expressed in mammalian cells; however, its function is unknown. The APP_{751} and APP_{770} isoforms both contain a 19 amino acid sequence corresponding to a Kunitz-type protease inhibitor in the extracellular region.[37]

There appears to be at least three major pathways for the metabolism of APP. The first involves a cleavage in the extracellular region, but within the β-amyloid sequence, releasing a soluble extracellular fragment denoted as APPs. This pathway would preclude the formation of β-amyloid.[38] The enzyme responsible for this cleavage is as yet unidentified but has been referred to as "α-secretase". The enzyme appears to act at a specific distance from the membrane and appears to prefer the presence of a bulky hydrophobic amino acid residue beside the peptide bonds it cleaves.[39] There are data to support at least two roles for APPs. First, APPs is identical to Protease Nexin II. Protease Nexin II is important for regulating proteases in the extracellular environment.[40]

Secondly, APPs found on the surface of platelets also plays a role in the clotting cascade as an inhibitor of activated coagulation factor XI. Platelets stimulated with thrombin secrete a protein functionally and immunologically identical to APPs.[41]

The presence of a consensus sequence (amino acids 759-762 using APP_{770} numbering) in the carboxy terminus of APP, known to mediate the endocytosis of cell-surface proteins via clarithin-coated pit internalization, raised the possibility that intact APP could be internalized, suggesting a second pathway. Labelling membrane-bound APP with biotin or antibodies to the extracellular region showed the peptide could be re-internalized to endosomes or lysosomes.[42] There peptide fragments containing the intact β-amyloid sequence were recovered. Treating cell cultures with chloroquine, an inhibitor of intracellular vesicular acidification, increased the half-life of APP. This suggested that proteolysis of internalized APP had occurred in an acidic intracellular compartment.[43] However intact β-amyloid has not been detected intracellularly, even within purified lysosomal preparations from cells expressing APP.

The third pathway was deduced by experiments that showed a second possible cleavage site of intact APP at precisely the amino terminus of β-amyloid yielding a shorter APPs segment and leaving a potentially amyloidogenic fragment behind (Figure 2). This second extracellular cleavage site has been called the "β-secretase" site.[44] Investigators also reported finding intact soluble β-amyloid released into cell culture as well as the detection of soluble β-amyloid in the cerebrospinal fluid (CSF) of both Alzheimer's disease patients as well as normal controls.[45] The presence of soluble β-amyloid in CSF of non-demented controls indicates that β-amyloid can be produced in the process of normal metabolism and does not require membrane damage. It has been proposed by some that β-amyloid could be produced independently of APP breakdown. However, evidence suggests that β-amyloid is produced during the catabolism of APP and not by local translation of APP mRNA.[46]

If formation of plaques from circulating β-amyloid is the initial insult in Alzheimer's disease, it is important to understand in greater detail the pathways leading to its production. One potential pathway in the nervous system involves the activation of a membrane-bound enzyme called phospholipase C (PLC). Activated PLC in turn cleaves a specific membrane-associated phospholipid named phosphatidylinositol 4,5-bisphosphate (PIP_2). Upon cleavage, PIP_2 is split into two second messengers, 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate ($InsP_3$). $InsP_3$ can stimulate the release of calcium from intracellular storage sites which in turn has various effects on cellular metabolism. The other second messenger, DAG, activates a membrane-bound protein kinase named protein kinase C. Protein kinase C when activated phosphorylates specific serine and

threonine residues in target proteins. The specific effects of protein kinase C depend on the specific proteins phosphorylated within that area of a cell.

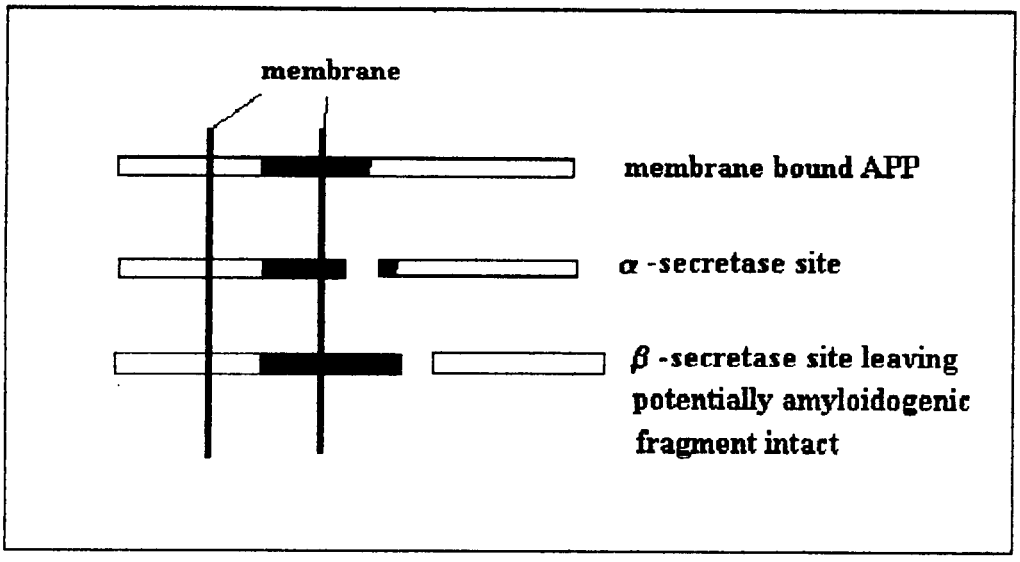

Figure 2: Enzymatic cleavage of amyloid precursor protein

Treatment of cell cultures expressing APP with phorbol esters, natural products which activate protein kinase C independently of DAG, or with okadaic acid, which inhibits protein phosphatases 1 and 2a, have resulted in increased secretion of APPs and reduced secretion of β-amyloid versus controls, implying phosphorylation plays some role in metabolism of APP. The authors note that alterations of potential phosphorylation sites in the APP molecule had no effect on the stimulation of secretion APPs by protein phosphorylation.[47]

Muscarinic receptors m_1 and m_3 are linked to phospholipase C. In support of the previous experiment, agents that activate muscarinic receptors m_1 and m_3 also increase APPs secretion and reduce β–amyloid secretion.[48] Activation of PLC not only activates protein kinase C by formation of DAG but can also increase cytoplasmic calcium levels through the action of $InsP_3$. To explore the question as to whether increased release of calcium could also affect APP metabolism in the same manner Greengard and colleagues conducted the following experiments.[49] Cell culture lines expressing the m_1 or m_3 receptors were incubated with phorbol esters for 17 hours, a treatment which has been shown to down-regulate protein kinase C. Addition of muscarinic agonists to these cultures still increased the formation of APPs and decreased the formation of β-amyloid,

even though treatment of the same cultures with phorbol esters now had no effect on APPs secretion. This implies that the $InsP_3$/calcium pathway was sufficient to mediate these effects on APP metabolism. In addition these cultures were treated with a compound that inhibits the uptake of calcium into the endoplasmic reticulum, thereby increasing the cytoplasmic calcium concentration. This also had the effect of increasing APPs release and decreasing β-amyloid release. This effect was still present even in cultures where protein kinase C had been down-regulated.

Another group of compounds that has shown an effect on APP metabolism are estrogens. Physiological concentrations of 17β-estradiol also has been shown to increase the secretion of APPs from cell culture. There was no corresponding increase in the production of APP, indicating the effect of estrogen may be to increase the secretion of APPs.[50] These experiments demonstrate that it may be possible to alter the metabolism of APP toward non-amyloidogenic pathways. The mechanism by which protein phosphorylation diverts APP metabolism from potentially amyloidogenic pathways is still undetermined but could involve phosphorylation of the "α-secretase" molecule. Alteration of putative phosphorylation sites on the APP molecule itself seems to have no effect on the ratio of APPs to β-amyloid secreted in the above experiments. Repeating these experiments using the transgenic mouse model for Alzheimer's disease mentioned earlier should demonstrate whether they have any *in vivo* effects on altering plaque formation.

Levels of circulating β-amyloid in the range of 3-5nM in the CSF have been reported.[51] So far no significant differences in CSF levels have been reported between Alzheimer's disease patients and non-demented controls. It is also not known whether significant alterations in β-amyloid occur more commonly in the elderly. It is conceivable that β-amyloid levels could actually decline in the face of active plaque formation within the brain. β-amyloid has been reported bound in CSF to a transport protein, transthyretin, that also transports thyroxin and vitamin A in the brain.[52]

The mechanism by which β-amyloid is cleared is unknown. Treating cultured mammalian cells with a variety of proteinases demonstrated that those from the metallo, aspartyl, and thiol classes reduced the levels of soluble β-amyloid remaining in the medium. Inhibitors to each class of proteinases could partially block β-amyloid degradation, and the combination of all three were able to block the degradation of β-amyloid completely. A 57 amino acid domain from APP containing the Kunitz-type protease inhibitor prepared by recombinant methods was able to reduce β-amyloid degradation by 50%.[53] This last finding could have significance for Alzheimer's disease. Isoforms of APP containing the proteinase inhibitor could retard clearance of β-amyloid thereby increasing its local concentration.

Tau

Neurofibrillary tangles (NFTs), the second major lesion of the brain found in Alzheimer's disease, are located in cell bodies and apical dendrites. Although NFTs have been reported to be a better correlate for pathological staging of the disease than are senile plaques, it is unclear if they are the initial lesion or a secondary response to nerve cell injury.[54] Ultrastructurally these lesions contain paired helical filaments (PHFs) ranging in width from 20nm at their widest point to 8nm at their narrowest. PHFs are composed of tau, an element of the neuronal cytoskeleton which is abnormally phosphorylated.[55] Tau is one of the microtubule-associated proteins (MAPs). Tau is found almost exclusively in axons, whereas other MAPs (e.g. MAP2c) are only found in cell bodies and dendrites. Tau binds to microtubules at tandem repeats of 31-32 amino acids located in the carboxy terminal half of the protein. Depending on the isoform there may be either 3 or 4 tandem repeats.[56] The reversible phosphorylation and dephosphorylation of tau regulates its binding to microtubules. In support of this it has been shown that dephosphorylated PHF tau can bind normally to microtubules[57]. A mouse model lacking the tau gene has been produced.[58] These tau(-) mice showed no obvious developmental abnormalities during development and were grossly indistinguishable from tau(+) mice. However upon examination by electron microscopy it could be shown that in some small caliber axons microtubule organization was disrupted. Microtubules were noted to run parallel for some distance with no tau crosslinks between them, and the total number and density of microtubules was also reduced. In addition the authors noted a two-fold increase in one of the other microtubule-associated proteins named MAP1 in tau(-) mice. The authors speculate that increased production of MAP1 can compensate for tau deficiency in large caliber axons but not in small caliber axons where tau makes up the primary microtubule-associated protein.

There are six different isoforms of tau, ranging in length from 352 to 441 amino acids, produced from a single gene by alternative mRNA splicing.[59] Tau expression is developmentally regulated. A single tau isoform (the 352 isoform) is expressed in fetal brain. All six are expressed in adult human brain. This isoform appears to be normally phosphorylated at more sites than adult tau.[60] In Alzheimer's disease the adult isoforms become maximally phosphorylated and self-associate in their MAP binding regions. Serine 202 is one site at which phosphorylation normally occurs in the fetal brain and abnormally in Alzheimer's disease brain.[61] All the phosphorylation sites are followed by a proline suggesting that protein kinases with a specificity for seryl-proline sites are responsible for phosphorylation under normal circumstances.[62] The serine-proline sites are spread

throughout the protein with the exception of the tandem repeat regions responsible for binding of tau to microtubules. One candidate kinase is mitogen-activated protein kinase (MAPK). Activation of this enzyme has been demonstrated to increase the phosphorylation of tau.[63] The phosphorylation state of tau, and thus its ability to bind to microtubules, is a balance between the actions of phosphorylases and phosphatases.

Decreased phosphatase activity in certain regions of the brain may be of greater consequence in Alzheimer's disease than increased phosphorylation. It has been shown that biopsied-derived tau from the brains of epileptics is phosphorylated at almost the same sites as PHF tau.[64] As time elapses the phosphatases in the tissue sections remain active and remove most of the phosphate groups. Phosphatases 1A and 2A activities were detected in the biopsied tissue. In contrast autopsy-derived PHF tau from Alzheimer's disease patients remains fully phosphorylated hours after death indicating a possible defect in phosphatase activity in those areas of the brain where PHFs form.

In affected nerve cells it is unlikely that tau is the only protein targeted for abnormal phosphorylation. For example another protein which is enriched in axonal growth cones and may be important for axonal proliferation and regeneration is known as GAP-43.[65] The relative phosphorylation of GAP-43 showed a 10-fold difference in the ratio of cytoplasmic/membrane phosphorylation in samples from the superior temporal gyrus of Alzheimer's disease brain between sections with high NFT density compared to low NFT density. [66]

Apolipoprotein E

One of the most important discoveries in Alzheimer's disease research was the finding that apolipoprotein E (apoE), a lipoprotein known to play a key role in cholesterol and triglyceride transport, was linked to the disease. ApoE is the primary apolipoprotein of the brain and is produced by astrocytes and microglia cells.[67] ApoE had been reported to be one of the constitutents of senile plaques,[68] and apoE from CSF was shown to bind to β-amyloid with high avidity.[69] A group of familial Alzheimer's disease patients had been described that had no linkage to chromosome 21, but had instead linkage to the long arm of chromosome 19.[70] The gene for apoE is located in the same region. ApoE is 299 amino acids long, with three common isoforms present in the general population. Apolipoprotein E3 (apoE3), the most common isoform, contains a cysteine at position 112 and an arginine at position 158. The other two isoforms, designated apoE2 and apoE4 differ from apoE3 by one amino acid. ApoE2, the least common isoform, contains cysteines at both

positions. ApoE4 has arginines at both positions.[71] These isoform differences have been shown to affect binding to the low-density lipoprotein (LDL) receptor. ApoE2 is defective in its binding to the LDL receptor and is associated with the lipoprotein disorder type III hyperlipoproteinemia.[72]

ApoE phenotype is inherited in a codominant fashion (Table 2).[73] The three common apoE alleles E4, E3, and E2 have frequencies in the general population of 15%, 77%, and 8% overall. Numerous positive and negative elements appear to regulate expression of apoE. The gene for apoE contains an estrogen response consensus element at positions -174 to -163 (relative to the transcription start site).[74] ApoE levels vary between the sexes and among post-menopausal women on estrogen replacement and those who are not by approximately 10%.[75] It is unknown what effect estrogen has on apoE expression in tissues that contain high levels of estrogen receptors.

Table 2: Percentages of apoE phenotypes

Phenotypes:	Prevalence (%)
E2/E2	1
E2/E3	12
E2/E4	2
E3/E3	60
E3/E4	22
E4/E4	3

An association between apoE phenotype and familial late-onset Alzheimer's disease was first reported in 1993.[76] The allele frequency of apoE4 in 30 Alzheimer's disease patients each randomly chosen from a different family was found to be 0.50 ± 0.06 while the allele frequency of apoE4 in 91 age-matched, unrelated controls was 0.16 ± 0.03. This association was also shown for sporadic Alzheimer's disease (i.e. disease where there is no known family history of Alzheimer's).[77] In 138 sporadic, probable Alzheimer's disease patients the apoE4 allele frequency ($\pm$ std. error) was 0.36 ± 0.042 compared to 0.16 ± 0.027 in the control group. A large autopsy study of 352 patients with sporadic disease showed an apoE4 frequency of 0.40 ± 0.026. Other studies confirmed these findings.[78,79,80]

Forty-two families with late-onset Alzheimer's disease were examined for the effect of increasing gene dose of apoE4 on the incidence of Alzheimer's disease, age at onset, and survivial.[81] The diagnosis was confirmed by autopsy in over 90% of cases. Twenty

percent of subjects with apoE2/E3 or apoE3/E3 were affected, 47% of the patients with apoE2/E4 or apoE3/E4 were affected, and 91% of patients who were apoE4/E4 had confirmed Alzheimer's disease. The risk of Alzheimer's increased by a factor of 2.84 for each additional apoE4. Similar results were seen on age at onset of Alzheimer's disease. Average age at onset was 84.3 years in subjects with no apoE4, 75.5 years in subjects with one apoE4, and 68.4 years in subjects who had two apoE4 alleles. Survival time also decreased with increasing gene dose of apoE4. It is important to note that not all patients in these series of studies with the apoE4 isoform developed Alzheimer's disease and patients with the apoE3 gene still were affected. This correlation between pathology and ApoE4 phenotype is not found found in Parkinson's disease.[82] Down's syndrome patients with the apoE4 phenotype do not develop Alzheimer's disease at an earlier age than do apoE3 carriers.[83] However, the apoE4 allele frequency is higher. The author suggests that in the presence of apoE2 Alzheimer's disease pathology seems to be induced more slowly.

There is great interest in determining what role apoE plays in the development of Alzheimer's disease and what potential avenues are opened to alter development and progression of the disease. It has been known for approximately a decade that apoE likely plays in a role in nervous system repair and regeneration.[84] Production of apoE is greatly increased following a crush injury to a peripheral nerve. ApoE appears to pick up cholesterol from cellular debris at the site and transport it inside the neuron via LDL receptors for use during subsequent axonal regeneration. The trigger for apoE production appears to be engulfment of cholesterol released from damaged cells by either microglia cells in the peripheral nervous system or astrocytes in the CNS.[85]

Apolipoprotein E has demonstrated isoform specific differences in binding to β-amyloid.[86] *In vitro* apoE4 binds in a shorter period of time and with a greater affinity than does apoE3 to β-amyloid. Increased plaque density has been reported in the cerebral cortex and hippocampus of late-onset Alzheimer's disease patients with one or two copies of apoE4.[87] ApoE4, as compared to apoE3, has also demonstrated *in vitro* binding differences to tau,[88] the constituent of neurofibrillary tangles, and to MAP2c another microtubule binding protein.[89] Binding studies using the different apoE isoforms and tau indicates that apoE4 demonstrates greatly decreased binding to tau than does apoE3 or apoE2, the reverse of that found in binding studies using apoE and β-amyloid. ApoE has been found inside neurons with and without NFTs.[90] One hypothesis about apoE4's role in Alzheimer's disease concerns its binding to tau.[91] In this model for the disease apoE4 causes Alzheimer's disease indirectly because it does not bind to the tandem repeat regions of tau, thus allowing tau to be abnormally phosphorylated and to accumulate into paired helical filaments. ApoE3 or E2 would protect tau from this abnormal phosphorylation. It

would be reasonable to assume that patients with one or two copies of apoE4 would have increased numbers of NFTs. One study has reported that NFTs did not differ statistically in number based on apoE phenotype.[92] Since the neuronal cholinergic system relies on a properly functioning phospholipid metabolism, it may be that individuals with apoE4 suffer increased age-related declines in this area of the brain due to some defect in lipid transport caused by apoE4. In cell culture apoE3 showed significantly greater effect on neurite extension than did apoE4.[93] Whatever the mechanism(s) apoE4 appears to play a key role. Analysis of apoE allele frequencies and age-adjusted prevalence rates for Alzheimer's show there are differences in these parameters among the world's populations. Interestingly, as the frequency of the apoE4 allele increases, the prevalence of Alzheimer's disease increases proportionally.[94]

Acetylcholine

The nucleus basalis of Meynert, the diagonal band of Broca, and the medial septum of the basal forebrain provide cholinergic projections to the cerebral cortex, hippocampus, and the amygdala.[95] The basal forebrain area appears to be important for learning and memory.[96] Antagonists of acetylcholine (Ach) can produce cognitive deficits similar to those of Alzheimer's disease.[97] One of the most consistent neurochemical changes in Alzheimer's disease brain is a loss of cortical and hippocampal cholinergic innervation with atrophy and often loss of the basal cholinergic neurons that project to the cortex and hippocampus.[98] Ach exerts its effects on the brain through two distinct classes of receptors, the muscarinic and nicotinic receptors.

Regulation of the synthesis and release of Ach in the normally functioning brain is a complex process. Determining alterations in an ongoing disease process such as Alzheimer's disease is even more complicated. An important precursor of neuronal Ach is choline, the majority of which is obtained from the circulation. A sodium-coupled choline transporter exists (Km=1-5μM) to deliver choline into cells which is then utilized by cholinergic neurons for two purposes, to produce phosphatidylcholine for membrane synthesis (phosphorylation) and to produce acetylcholine as a neurotransmitter (acetylation).[99,100]

Ach is synthesized from acetyl CoA and choline in a reaction catalyzed by choline acetyltransferase (ChAT) which is an enzyme existing in membrane and cytoplasmic forms. The membrane form can be activated by increased neuronal activity enabling neurons to maintain adequate neurotransmitter production.[101] If cholinergic neurons are

provided with very low levels of choline and are repeated depolarized, the neurons are able to shift from phosphorylation of choline to acetylation.[102] This allows cholinergic neurons to continue to synthesize Ach for neurotransmission. If cholinergic fibers are maintained in a choline-free solution they continue to make large amounts of acetylcholine. Under these conditions there is a significant decrease in phosphatidylcholine content of the membranes.[103] This could imply that cholinergic fibers were catabolizing phosphatidylcholine from membranes in order to obtain choline for production of neurotransmitter. In support of this elevated levels of phospholipid metabolites have been isolated from the temporal cortex of Alzheimer's disease brains.[104] As the number of neurons in these areas decline with aging, other cholinergic neurons may increase their rates of activity to compensate. This could lead to membrane damage as the neurons scavenge choline from phospholipids and further cell injury.

Neurotrophins

There is certainly evidence for a role for neurotrophins in the development and maintainance of cholinergic neurons. At the present four members of this family are known, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5), which share about 50% amino acid homology overall and 87% homology in the tyrosine kinase domains.[105] The neurotrophins bind two classes of cell surface receptors, termed the high-affinity receptor ($K_d=10^{-11}M$) and the low-affinity receptor ($K_d=10^{-9}M$). The low affinity receptor, named p75, binds each of the neurotrophins approximately equally.[106] The functional role of this receptor is still unclear. Barker and Shooter have suggested that p75 may act to concentrate NGF locally in the microenvironment surrounding the receptor and thus enhance the ability of the nerve growth factor receptor to bind and respond to NGF.[107]

The other class contains the trk receptors which possess tyrosine kinase activity and bind their respective ligands with high affinity.[108] TrkA preferentially binds NGF, trkB binds BDNF and NT-4/5, and trkC binds NT-3. In addition trkB, trkC and p75 can exist as truncated receptors. Truncated trkB and trkC receptors lack the intracellular tyrosine kinase region. TrkA is localized to only a few neuronal cell types in the CNS and is not found on non-neuronal cell types in the brain. However it may play an important role in Alzheimer's disease as it is estimated the majority of the NGF-responsive neurons are cholinergic. TrkB and C have a wide distribution. The truncated forms of trkB has been

found on non-neuronal cell such as astrocytes. Truncated receptors may provide a way to maintain high concentrations of a certain growth factor in a localized area (Figure 3).[109]

Neurotrophins are increased in the adult CNS in response to a wide range of brain insults including seizures, hypoglycemia, hypoxia, and focal lesions.[110] NGF, BDNF, and NT-3 have been shown to protect against metabolic and excitotoxic insults.[111,112] Transection of the fimbria, the main fiber bundle through which cholinergic neurons travel to reach the hippocampus, results in atrophy of those neurons and down regulation of ChAT. Infusion of NGF into the ventricles of the brain prevents these changes, presumably by interacting with receptors on the surface of the neurons, setting in motion a cascade of events leading to protection and/or regeneration.[113] In contrast neural cells that express p75 are more susceptible to the toxic effects of β-amyloid than otherwise similar cells that do not express p75.[114]

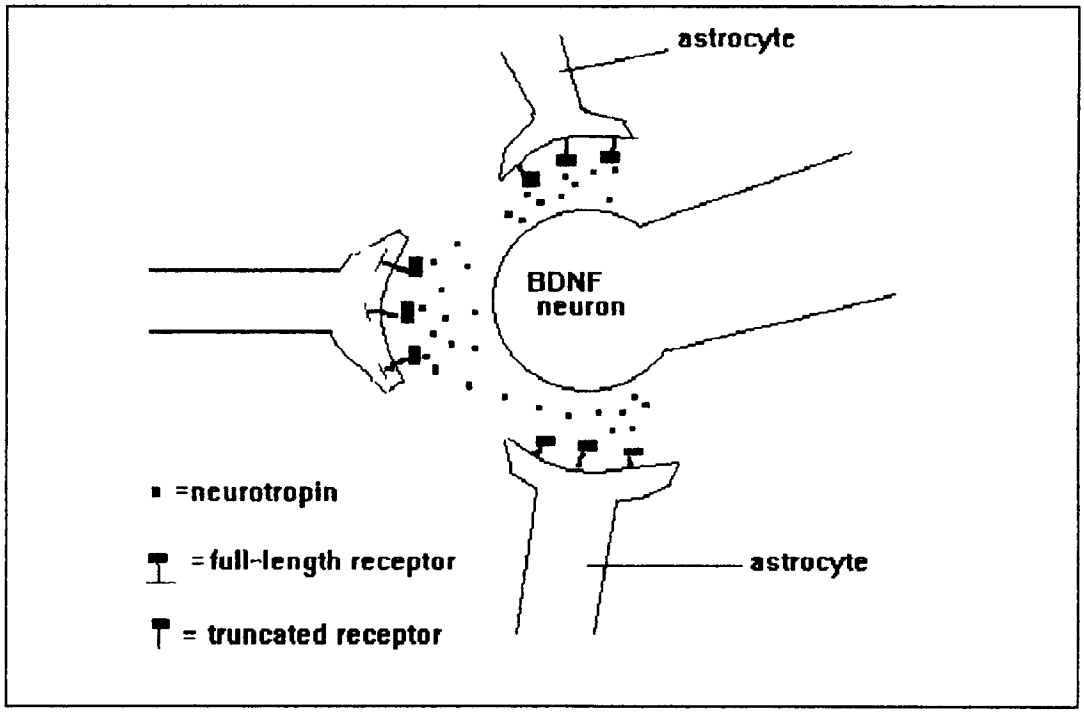

Figure 3: Possible role of full-length and truncated neurotropin receptors

Estrogens

Cholinergic neurons of the basal forebrain are targets not only of neurotrophins but of estrogen as well. In basal forebrain cholinergic cells estradiol induces the enzyme ChAT, which is the key enzyme for acetylcholine biosynthesis.[115] Estrogen receptors and the p75 nerve growth factor receptor have both been reported in cholinergic neurons of the basal forebrain.[116] Estrogen regulates the transcription of a wide variety of cellular proteins by interacting with estrogen receptors located in the cytoplasm. The receptor-estrogen complex then recognizes an estrogen response element in the promoter region of a particular gene and binds at that location. The promoter region of p75 and trkA contain sequences with a high degree of homology to the estrogen response elements.[117] Estrogen has been shown to upregulate trkA mRNA and downregulate p75 mRNA in adult dorsal root ganglia isolated from rats injected with a repository formulation of estradiol.[118] These effects were time dependent with an increase in ChAT activity occuring soon after estrogen exposure followed by effects on trkA and p75 several days later. These same neurons were shown to contain estrogen receptors. Similar increases in ChAT followed by decreases in p75 mRNA one week later were reported for rat basal forebrain cholinergic neurons but not striatal cholinergic interneurons.[119] Striatal cholinergic interneurons are not affected in Alzheimer's disease. These studies suggest an important interplay between estrogen and neurotrophins. Therefore a decline in either of these systems may adversely affect the health and maintainance of cholinergic neurons during aging.

In summary estrogen may play multiple roles in the daily maintainance of the central nervous system and in its response to injury. There may be multiple risk factors for the development of Alzheimer's disease including the decline in estrogen levels at menopause. If estrogen plays a role in the maintaining the proper functioning of cholinergic pathways in areas of the brain important for learning and memory and declining estrogen levels accelerate the amyloidogenic pathways of the APP, then a survey of post-menopausal females on estrogen replacement therapy (ERT) compared to age-matched controls may reveal a difference in incidence of SDAT between the two populations.

Materials and Methods

Study Populations

Study populations were drawn from two sources, representing ambulatory and institutionalized populations, and were analyzed separately. The primary study population was composed of members of the Oregon Public Employees Retirement System (PERS), (n=18,893). Information on these subjects was obtained from a newly created database combining information such as prescription drug records for members of the plan from July 1, 1988 to May 1995, outpatient and inpatient ICD-9-CM diagnosis codes from Medicare and PERS Medicare supplemental records, and descriptive information on each enrollee such as date of birth and gender. Only subjects with a diagnosis of senile dementa of the Alzheimer's type (SDAT) were placed in the study group. Individuals with other forms of dementia were excluded. The remaining subjects served as the control population. Only subjects 65 years of age and over were included in the analysis.

The second source came from a survey conducted on 118 long-term care (LTC) facilities within the state of Oregon. Data on the subjects were obtained from records of two consulting pharmacy services provided to the above facilities. A diagnosis of Alzheimer's disease was obtained by screening the patient's medical record upon admission to the respective facility and was based upon the ICD-9 code. In order to insure that the diagnosis of SDAT be as accurate as possible, patients admitted with a diagnosis of alcoholic dementia, organic brain syndrome, schizophrenia, hypoxia, or Parkinson's dementia, in addition to Alzheimer's disease, were excluded from analysis as well as patients diagnosed as "possible" Alzheimer's, or "multi-infarct dementia vs Alzheimer's disease. A total of 3141 cases were included for analysis.

Drug Data Set

The prescription drug program records for the PERS set contain information on dates of service, National Drug Code (NDC) number, and quantity of medication actually dispensed on each date of service. Nearly all prescription drugs are covered under the benefit, and out-of-plan utilization is considered by plan administrators to be very small.

Numerous estrogen replacement products are available in a variety of dosage formulations and manufacturers. It would be difficult to accurately determine an absolute equivalency among these products. To control for differences in response due to this

potential variability we included only those patients receiving Premarin^R brand of conjugated estrogen. Of the 35,951 estrogen prescriptions filled for PERS enrollees since 1988, 80.4% were for Premarin^R brand products. Similarly only patients in the LTC set receiving Premarin^R brand of conjugated estrogen were included for final analysis. A total of 3450 females in the PERS data set and 190 females in the LTC data set received estrogen replacement therapy under these criteria.

Estrogen exposure was examined in the PERS set in four ways. First, an estrogen exposure was defined as a subject having received any prescription for an estrogen product during the study period of July 1988 through May 1995. Four subjects were dropped from the PERS database because the date of their diagnosis for SDAT preceded the date of their first estrogen prescription. Second, the actual dose prescribed for a patient from 0.3mg up to 2.5mg was examined, to determine if the odds ratio for SDAT decreased with increasing dose. If a patient received prescriptions over the course of the study period for more than one strength the highest strength used for the longest time was selected. Third, the estrogen exposure in dose-years was determined. A dose-year was defined as one dose per day for 300 days per year. Fourth was a measure of the cumulative dose, which was determined by multiplying the dosage strength for each individual prescription by the number of tablets received and summing those over time. Exposure for patients receiving conjugated estrogen vaginal cream was determined as follows. Since systemic levels can be achieved with topical application of the creams, these products were included as an exposure. The vaginal cream is available as 0.625mg/gm in either a 43 or 45g tube with an applicator graduated to deliver between 1-4g. To determine days of therapy and cumulative dose we assumed a 2gm application per daily dose or approximately 20 days per tube dispensed. In the LTC set to be considered as an estrogen exposure patients had to be receiving estrogen replacement therapy on admission to the respective facility. It was not possible to accurately determine the actual length of estrogen exposure prior to admission, and this was not considered as a variable in subsequent analysis of this group. A total of 190 females met these criteria in the LTC set for estrogen exposure.

The elderly take, on average, more prescription drugs than any other segment of the U.S. population. It is possible that if estrogen replacement therapy (ERT) does reduce a woman's risk of developing Alzheimer's disease, that effect may be altered by concominant administration of other prescription medications. Four classes of medications were selected to test as possible confounders.

Progesterones are often co-prescribed with estrogen replacement therapy to reduce the incidence of endometrial hyperplasia. In the PERS data set 29% of estrogen users also received a progesterone product. The second class of drugs were the hydroxymethylglutaryl-CoA (HMG-CoA) reductase inhibitors, compounds approved to reduce serum cholesterol levels. Overall 6.9% of women also had a prescription for these agents and 8.6% of women on ERT also had prescriptions filled for HMG-CoA reductase inhibitors. HMG-CoA reductase inhibitors have been shown to lower serum levels of apolipoprotein E (apoE). One phenotype of apoE, apoE4 has been shown to be an important risk factor for Alzheimer's disease. The third class are the non-steroidal anti-inflammatory agents (NSAIAs). NSAIAs are commonly used for arthritic pain, a common complaint in the elderly. Inflammation has been postulated to be part of the cause of neurological damage in Alzheimer's disease.[120] NSAIAs have been reported in one trial to have a beneficial effect in SDAT.[121] Finally medications that may act to increase the metabolism of estrogens may reduce their overall effect. Drugs that induce liver microsomal enzymes, such as carbamazepine and barbiturates were taken by 1.8% of women in the PERS set on ERT. These classes of medications will be examined as confounders.

Data Analysis

Logit analysis can be applied in comparative studies of this design and is considered an appropriate method of analysis. The software program *CSS.StatisticA* (StatSoft, Inc., Tulsa, OK 74104) was used to model the data. The results are reported are changes in the odds ratio for SDAT with 95% confidence intervals.

Results

For the PERS sample the mean age of males was 74.9 (SD=6.6) and of females 76.7 (SD=7.4) years respectively. The mean age in the LTC sample was 85.2 (SD=8.2) years for males and 85.0 (SD=9.9) years for females. The average age of estrogen users was 74.9 years versus 77.6 years for non-users overall in PERS. For females with SDAT, estrogen users had an average age of 81.1 years as compared to 84.6 years for non-users. A breakdown of subjects in both groups by age, gender, and SDAT is described in Table 3.

Table 3: Breakdown of subjects by age, gender, and SDAT.

	AGE RANGE			
	65-74 YRS	75-84 YRS	>84 YRS	TOTAL
PERS				
FEMALES	4663	4010	1751	10424
FEMALES SDAT	11 (0.2%)	53 (1.3%)	59 (3.4%)	123 (1.2%)
MALES	4586	2993	807	8469
MALES SDAT	13 (0.3%)	34 (1.1%)	36 (4.5%)	83 (1.0%)
LTC				
FEMALES	290	881	1407	2578
FEMALES SDAT	20 (6.9%)	121 (13.7%)	197 (14.0%)	338 (13.1%)
MALES	86	203	288	577
MALES SDAT	7 (8.1%)	38 (18.7%)	31 (10.7%)	76 (13.2%)

The odds ratio of SDAT in females was not significantly different than in males after accounting for age (Chi^2=0.404, p=0.55). This was also true in the LTC set (odds ratio= 0.98, 95% CI= 0.72 to 1.35).

During the study period 39,591 prescription were filled for PERS enrollees, 80.4% of which were for Premarin^R brand of conjugated estrogen. Only 2% of the conjugated estrogen prescriptions were for vaginal creams. Estrogen usage was highest in the 65-74 year old age range (40.1%) and lowest in the over 84 year old group (16.0%). The 0.625mg strength of conjugated estrogen was the most frequently prescribed strength,

being used by 71.2% of women on ERT, the 0.3mg strength accounted for 18.3%, and the 1.25mg strength accounted for 8.9% of all prescriptions (Table 4).

Table 4: Breakdown of conjugated estrogen dose by age (PERS).

DOSE	65-74 YRS	75-84 YRS	>84 YRS	TOTAL
0.3mg	302	263	67	632
0.625mg	1364	898	197	2459
0.9mg	31	11	1	43
1.25mg	186	105	16	307
2.5mg	8	1	0	9
TOTAL	1891	1279	284	3450

The length of ERT in years steadily declined from 39% receiving ERT for less than one year down to 4.2% receiving ERT for greater than five years. Sixty percent received conjugated estrogen for two years or less (Table 5). Of those patients using topical conjugated estrogen cream, 87.1% received treatment for less than six months while only 3.3% received treatment for more than one year.

Table 5: Length of conjugated estrogen replacement therapy in years (PERS).

	<1 yr	1-2 yrs	2-3 yrs	3-4 yrs	4-5 yrs	5-6 yrs	6-7 yrs
Controls	1350	731	525	397	285	119	27
SDAT	5	4	3	2	1	1	0
TOTAL	1357	738	529	399	286	120	27

Among estrogen users in the PERS set only 0.47% were reported to have SDAT compared to 1.6% among non-users (Table 6). This difference was highly significant ($Chi^2 = 22.68$, $p = 0.000002$). However, the difference of SDAT between estrogen users and non-users in the LTC set was not significant ($Chi^2 = 1.2$, $p = 0.27$) (Table 7).

Table 6: Breakdown of estrogen users and non-users by age and diagnosis (PERS).

	NO ESTROGEN (PERS) SDAT		ESTROGEN (PERS) SDAT	
	NO	YES	NO	YES
65-74 YRS	2765	7	1887	4
75-84 YRS	2686	46	1271	7
> 84 YRS	1416	54	276	5
TOTAL	6867	107	3434	16

Table 7: Breakdown of estrogen users and non-users by age and diagnosis (LTC).

	NO ESTROGEN (LTC) SDAT		ESTROGEN (LTC) SDAT	
	NO	YES	NO	YES
65-74 YRS	237	19	29	1
75-84 YRS	673	112	84	9
>84 YRS	1149	187	56	10
TOTAL	2059	318	169	20

The final model included age, and indicator variables for NSAIAs and medications classified as enzyme inducers. Estrogen exposure in the PERS group was found to be significant (Chi^2, drop in deviance test = 5.95, p=0.01). The odds ratios for estrogen exposure and daily dose were 0.51 and 0.73 respectively (Table 8) as compared to estrogen non-users. Odds ratios for length of treatment and relative cumulative dose are also described in Table 8.

Table 8: Odds ratios with 95% confidence intervals for estrogen use variables for SDAT.

PARAMETER OF ESTROGEN USE	ODDS RATIO	95% C.I.
PERS		
Exposure	0.51	0.26-0.97
Daily Dose	0.73	0.53-1.01
Duration estrogen ≤ 2 YRS	0.44	0.19-1.02
Duration estrogen > 2 YRS	0.63	0.24-1.66
Cumulative dose ≤450mg	0.48	0.20-1.13
Cumulative dose >450mg	0.55	0.22-1.33
LTC		
Exposure	0.66	0.33-1.30
Brenner, et al 1994 Exposure (Y/N)	0.70	0.4-1.50
Paganini-Hill, et al 1994 Exposure (Y/N)	0.67	0.38-1.17

Discussion

The Oregon Public Employees Retirement System (PERS) data set contains a population of post-menopausal women currently on ERT at a much higher overall rate than the national average (33% vs 10%). In the 65-74 year old age group the percentage of women on ERT is just over 40%. It is also of interest to note the low numbers of reported Alzheimer's disease cases in this population. A recently reported study on the age-specific incidence rates of Alzheimer's disease reported a cumulative incidence rate of approximately 10% by age 75 years.[122] The prevalence rate in females in PERS was 1.2% overall and only 0.24% in the 65-74 year old age group, the group which also had the highest percentage of ERT. The percentages of ERT in women with SDAT (13%) versus controls (33%) paralleled the differences reported by other investigators.[123] In addition the overall length of conjugated estrogen treatment and relative cumulative dose were also greater in controls than in SDAT (data not shown).

In addition to its large sample size, this study is unique in restricting estrogen exposure to one brand of conjugated estrogen, making interpretation of estrogen exposure less subject to inter-product variability. The combination of ambulatory and institutionalized patients also more closely approximates the mix of patients seen in society at large.

Our study contains weaknesses which should be borne in mind when interpreting the results. The diagnosis of SDAT was obtained from Medicare billing. For those patients so diagnosed there was no follow-up autopsy results to confirm the accuracy of the diagnosis. Previous studies have revealed accuracy of diagnosis rates of 80-95% when confirmation of the initial diagnosis of Alzheimer's disease was possible following an autopsy. It was not possible to determine from the available data whether the diagnosis of SDAT was made by a neurologist using standardized criteria and confirmed at follow-up office visits. Since up to 20% of the patients reported to have SDAT may have another form of dementia, the distribution of estrogen users between those correctly and incorrectly diagnosed as SDAT may affect the calculated odds ratio. Of the 64 females diagnosed as SDAT in the 65-84 year old age range, as many as 13 may have another form of dementia. Eleven of these 64 women received ERT during the study period. The estrogen breakdown between the accurately and the inaccurately diagnosed groups would be expected to be 80:20. If, in our sample, more of the ERT was received by the incorrectly diagnosed patients, then the odds ratios reported here are conservative as the

actual estrogen exposure overall in the SDAT group would be reduced. Conversely the probability of obtaining a sample in which all the ERT ended up in the group correctly diagnosed as SDAT is about one in ten (actual probability =0.10, Fisher's Exact Test).

The other potential problem related to diagnosis is the possibility of under reporting of patients with early mild disease and its temporal relationship to ERT. Thus missed diagnosis of early disease could be a significant factor. The very low rates of reported cases of SDAT in the PERS group could be due to under reporting rather than a response to higher than average rates of women on ERT.

Patient non-compliance with medication regimens is always possible as is the possibility that patients may obtain medications from sources outside of the medication plan. In 1991 75.9% of PERS enrollees used the prescription drug benefit at least once. This is comparable to usage in 1987 among all U.S. elderly, 80.7% used at least one prescription drug during the year. Plan administrators believe that because of the favorable pricing, large number of pharmacy participants, and coverage of almost all prescription medications under the plan usage outside the plan is very low.

The role of apolipoprotein E4 (apoE4) as a risk factor for the development of Alzheimer's disease may also have influenced our results. We cannot identify individuals with an apoE4 phenotype and this may be an important explanatory variable in the model. Patients with one or two copies of apoE4 allele develop Alzheimer's disease more frequently and at an earlier age than do those with no copies of apoE4. It would be of great interest to know how ERT may affect odds ratios for individuals with different apoE phenotypes.

It was also of interest to note the role of other medications as possible confounders of estrogen's effects on the brain. Indicator variables for NSAIAs and medications such as phenobarbital and carbamazepine were included in the final model. There has been interest in use of mediators of inflammation in slowing the development of Alzheimer's disease. Recent reports suggest inflammatory processes may play a role in the development of neurological damage in Alzheimer's. NSAIAs were used by 48.5% of women on ERT during the study period compared to just 29% of estrogen non-users. Medications such as carbamazepine and phenobarbital may induce liver microsomal enzymes and lower serum estrogen levels possibly reducing its effectiveness. However, only 1.8% of women receiving ERT also had prescriptions filled for these medications. These medications are most often used to manage seizure disorders. There is currently no information known to us on the rates of SDAT in epileptics or whether those rates are greater than the general population. We found no effects of progesterones in altering the odds ratio for SDAT in patients receiving ERT.

Our results demonstrate a significantly lower odds ratio for SDAT in women receiving ERT and in those women receiving higher daily doses of conjugated estrogen (Table 8). However the hypothesis that a decline in estrogen levels following menopause may result in CNS pathology, and that pathology may be prevented or delayed by ERT would be difficult to defend if the more important variables describing estrogen therapy, such as length of treatment and total cumulative dose, did not significantly alter the odds ratio for SDAT. Our results reveal a reduced odds ratio for women who received ERT for two years and less during the study period. It should be borne in mind that entry of patients into the PERS system occurs at age 65 years, thus this is a relative measure of length of treatment. The odds ratios also were reduced for women taking ERT for greater than two years, but the reduction in odds is much less. It is possible that although estrogen replacement therapy does initially reduce the odds of developing SDAT, that effect declines with time. The reduction in odds with higher cumulative dose is very small and may indicate that at conjugated estrogen doses used clinically the lower doses are sufficient to provide a protective effect. The low number of women reported as SDAT patients also on ERT may have made a clear-cut interpretation of these variables and their influence on the odds of SDAT more difficult.

Our results do agree with two previously published articles by Paganini-Hill and Henderson[124] and Brenner, et al.[125] (Table 8), and indicate a decreased odds ratio for SDAT in post-menopausal women receiving ERT. Our proposal anticipated a larger subset of the PERS population with a diagnosis of SDAT, but even with the resulting smaller SDAT subset, exposure to conjugated estrogen was still a significant factor in the odds of SDAT among postmenopausal females. Whether prophylactic treatment with conjugated estrogen in postmenopausal females will postpone the onset of SDAT, and, if so, what the appropriate dose might be, what the optimal duration of therapy might be, or which patients are most likely to benefit is not clear from this or any published study to date. Given the scope of the problem further research using larger sample sizes, including apoE phenotype, confirming accuracy of diagnosis, and possibly prospective trials is indicated.

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