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EVALUATION OF HOW CIGARETTE SMOKE IS A DIRECT RISK FACTOR FOR ALZHEIMER'S DISEASE

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Abstract

Cigarette smoking is a risk for Alzheimer's disease (AD), the pathological hallmark of which is amyloid- β ($A\beta$) brain deposits. We found the adjusted risk of AD was significantly increased among medium level smokers (RR = 2.56; 95% CI = 1.65–5.52), with an even higher risk in the heavy smoking group (RR = 3.03; 95% CI = 1.25–4.02). This systematic review and original data further support this association. We searched Pubmed, Google scholar, and PsyINFO for original population study articles, meta-analyses, and reviews published between 1987 and 2011. Some studies were excluded due to design flaws including survivor bias. We performed analyses of: 1) amyloid precursor protein (APP) processing in N2a cells overexpressing Swedish mutant APP (SweAPP N2a) exposed to cigarette smoke condensate (CSC), 2) microglial inflammatory response to CSC, and 3) CSC exposed microglial phagocytosis of $A\beta_{1-42}$. CSC significantly promotes neuronal $A\beta$ generation, increases microglial IL-1 β and TNF- α production, and decreases microglial $A\beta_{1-42}$ phagocytosis. The mechanism underlying the epidemiological association of cigarette smoking with AD might involve the effect of cigarette smoke on APP processing, a reduction of $A\beta$ clearance by microglia, and/or an increased microglial proinflammatory response. In vivo studies are required to fully elucidate how cigarette smoke promotes AD.

Keywords

Cigarette; Smoking; Alzheimer's disease (AD); Dementia; Risk; Amyloid- β ($A\beta$)

CIGARETTE SMOKING AS A RISK FACTOR FOR PROMOTION OF ALZHEIMER'S DISEASE

In 2006, there were 26.6 million people with Alzheimer's disease (AD), and this number is predicted to quadruple by the year 2050 (7). As such, an understanding of the mechanisms underlying this phenomenon is important. What follows is a brief background systematic review of the available literature that is appropriately designed without survivor bias, or tobacco company sponsorship. We will then move on to discuss a possible molecular mechanism underlying this epidemiology.

There is much evidence to indicate that cigarette smoking has a dose-dependent association with AD development (1,8,25,28,35,36,39,40). One of the first reports to identify cigarette smoking as a risk for the development of AD was published some 25 years ago. This was a case control study in which 98 men with clinical AD were compared to 162 controls matched by age and town of residence. AD cases were positively correlated with number of cigarettes smoked, although the result was not significant (47).

Ten years later, in a similar case-control study of 98 AD patients and 97 age- and sex-matched controls, daily smoking showed a trend toward an increase risk for AD [45.9% vs. 26.5% in non-smokers; odds ratio (OR) = 1.73, $p = 0.08$] while light smoking seemed to have a protective effect (56). A 1998 seminal report in the *Lancet* revealed that smoking was associated with a doubling of the risk of dementia and AD by Cox proportional hazards regression, after adjustment for age, sex, education, and alcohol intake [current smoker vs. never smoker, relative risk (RR) 2.3, 95% CI 1.3–4.1] (35). They first found which association between smoking and AD development was modified by apolipoprotein $\epsilon 4$ (apoE4) allele, which was a well-known genetic risk factor for incident AD (45).

Interestingly, current smokers without an apoE4 allele had significantly increased AD risk of 4.6 (95% CI 1.5–14.2). Those carrying the apoE4 alleles had a nonsignificantly increased risk of AD (RR 0.6, 95% CI 0.1–4.8). This may indicate that the risk conferred by the ApoE4 gene is so strong that cigarette smoking only has a negligible effect. The following year Merchant and colleagues (32) found that relative risk of AD among current smokers was almost double the control (RR 1.95, 95% CI 1.2–3.0). Consistently it was found that smokers had the highest risk of AD (RR 2.1, 95% CI 1.2–3.7) compared to never smokers in absent apoE4 allele participants; however, among carriers of apoE4 current smoker decreased in relative risk (RR 1.4, 95% CI 0.6–3.3). Interestingly, they found a trend among former smokers, who had a slight nonsignificant reduction in AD incidence as the relative risk was 0.7 (95% CI 0.5–1.1). This may be explained by the notion that those surviving former smokers may have more effective DNA repair mechanisms (5,33). As a consequence, the accumulation of aging-associated defects in DNA and DNA repair, which are presumed to be associated with AD, may be altered among surviving former smokers, reducing their susceptibility to AD (32).

In further support, in a pooled analysis of four prospective studies with 28,768 person-years of follow-up, it was found that current smoking significantly increases the risk of AD (28). Specifically, current smokers had a significantly increased relative risk of AD of 1.74 (95% CI 1.21–2.50) compared with never smokers. Tyas and colleagues (52) studied the association between smoking and AD and other types of dementia in the Honolulu Heart Program (1965–1971) and follow-up assessment for dementia (1991–1996) of 3,734 Japanese-American men (29). Neuropathologic data were available from 218 men. There was no significance increased in risk of AD incidence in current smokers (RR 1.0, 95% CI 0.61–1.63). However, the risk of AD in smokers increased with pack-years of smoking at medium (OR 2.18, 95% CI 1.07–4.69) and heavy levels (OR 2.40, 95% CI 1.16–5.17). This

study found a lack of association between very *heavy* smoking and AD. This is probably explained by the hardy survivor bias effect, which will be expanded upon in the next section of this article (52). Upon autopsy in this study it was found that the number of amyloid plaques increased with amount smoked (52). Indeed, deposition of these amyloid- β peptide (A β) as β -amyloid plaques is a pathological hallmark of AD. They are accompanied by increased activation of microglia-mediated inflammatory responses (44).

We also conducted a prospective 2-year study of 2,820 persons aged 60 or older and found current smokers had significantly increased risk of AD (RR 2.71, CI 95% 1.63–5.42). When compared to light smokers, the risk of AD was significantly higher among smokers with a medium level of exposure (RR 2.56, 95% CI 1.65–5.52), with even greater risk of AD in the heavy smokers (RR 3.03, 95% CI 1.25–4.02) (25), adjusted by age, sex, education, blood pressure, and alcohol intake. This study further suggests that both smoking status and its intensity are associated with AD (25). In 2007, Reitz and colleagues published results from the Rotterdam study, a prospective cohort study of 6,868 subjects, 55 years or older and free of dementia at baseline studied over an average of 7.1 years. It was found that current smoking increases the relative risk of AD by 1.56 (95% CI 1.21–2.02). Moreover, in non-apoE4 carriers, current smoker increased in risk of AD incidence as well (RR 1.95, 95% CI 1.29–2.95) (39).

One potential explanation for nonassociation between smoking and AD in apoE4 carriers is that smoking may be harmful through alternative mechanisms, but beneficial in apoE4 carriers. This hypothesis is supported by previous findings that apoE4 carriers with AD have fewer nicotinic receptor binding sites and lower activity of choline ace-tyltransferase compared with noncarriers (35,37). Finally, Peters and colleagues (36) did a meta-analysis from 1996 to 2007 of individuals aged 65 and over, and concluded that current smoking is a significant risk for AD (RR 1.59, 95% CI 1.15–2.20).

In 2010 and 2011, two reports further reinforced the positive association between cigarette smoking and AD. In a meta-analysis by Cataldo and colleagues (8), smoking was a significant risk factor for AD after accounting for bias introduced by author tobacco industry affiliation and survival bias phenomenon in case-control studies. After controlling for study design, quality, secular trend, and tobacco industry affiliation, a significant increase in AD risk was associated with cigarette smoking for cohort studies of average quality in 2007 (1.45, 95% CI 1.16–1.80). This review also indicated case-control studies tended to demonstrate lower risks than cohort studies and the majority of the tobacco industry affiliated studies used case-control design (RR 0.60, 95% CI 0.27–1.32) (8). After controlling for study design, tobacco industry affiliation was independently associated with lower risk estimates. In contrast to the significant increase in risk of AD development in non-tobacco industry-associated studies (1.72 ± 0.19 , $p < 0.0005$), the combination of case-control design and tobacco industry affiliation yielded a protective odds ratio of 0.86 (95% CI 0.75–0.98). Again these putative “protective” effects of smoking cigarettes was generated by poor study design, not accounting for survivor bias, and possible conflicts of interest due to affiliation with the tobacco industry (8).

In a seminal report by Rusanen and colleagues (40), it was found in a cohort of over 21,000 individuals those smoking >2 packs/day had a significantly higher risk of AD than nonsmokers [adjusted hazard ratio (HR) 2.57, 95% CI 1.63–4.03]. They adjusted for age, sex, education, race, marital status, hypertension, hyperlipidemia, body mass index, diabetes type II, heart disease, stroke, and alcohol use (40). These most recent findings in this well-designed study are consistent with previous studies showing that heavier smoking is associated with greater AD risk (8,25,28,35,36,39,40).

THE CONTROVERSY OVER THE MYTH THAT CIGARETTE SMOKING IS PROTECTIVE FOR AD

Several publications have indicated that smoking might have no, or a protective, effect (14,30), particularly those of case-control design (26). However this conclusion is likely misleading due to selection or “survivor” bias and funding of the research by the cigarette industry (8,22,33).

Addressing the latter, empirical support for selection bias due to censoring by death in epidemiologic studies of the effect of cigarette smoking on risk of AD is provided in a recent review by Hernán and colleagues (22). The deleterious effect of smoking on mortality is most likely not reduced in the demented. Conversely, in the elderly, comorbidity can have an additive effect on mortality (20). If this phenomenon occurs with dementia and smoking, smokers who become demented are likely eliminated early from the population, thus resulting in an underrepresentation of demented smokers in cross-sectional samples as well (22,53). This is supported by observations that smoking is associated with an increased mortality among patients with dementia but not controls, suggesting that patients with dementia who have been smokers may be underrepresented in cross-sectional samples (55). This hypothesis is also consistent with findings of a much greater effect of smoking on mortality in demented (HR 3.4) than in nondemented (HR 0.8) subjects (55). For example, in a study of a cohort of 668 people (75–101 years) it was found that smoking was not associated with prevalent AD. However, over a 3-year period incidence of AD was higher in the smokers. Over a 5-year follow-up period, the mortality rate was greater in smoking demented (HR 3.4) than nondemented (HR 0.8) patients (55). Thus, this report suggested that smoking is not protective against AD, and the cross-sectional association is likely due, at least in large (10), to earlier mortality in the smoking group (26,55).

Indeed, a systematic review of 12 studies by Hernán and colleagues (600 subjects, adjusting for age and sex) revealed a wide range of relative risk of AD for smokers versus nonsmokers (0.27–2.72). They found that the minimum age at entry (55–75 years) accounted for much of the inconsistency between studies, and it was suggested that selection bias secondary to censoring by death was the main explanation for the reversal of smoking-induced risk of AD with increasing age (22).

Indeed, it is known that study subjects with complete follow-up are healthier and have better age-specific cognitive scores than those with incomplete follow-up, which is precluded by earlier death due to smoking. A well-known potential result of these differences is selection bias: when the analysis is restricted to individuals with complete follow-up (e.g., those not too ill to participate) (12). In this scenario, it is very possible to find an exposure–outcome association that is not due to the causal effect of the exposure on the outcome (21). An extreme example of “incomplete follow-up” for nonfatal outcomes such as AD is death; thus, censoring by death may introduce selection bias as in the study by Wang and colleagues (55). In studies of the elderly (those at highest risk of developing AD), this selection bias may be quite significant as the death rate is high and mortality is often affected by the exposure (19)—in this case, cigarette smoking.

In earlier meta-analyses of case-control and cohort studies by Almeida et al. (3), similar conclusions were drawn. When the authors restricted their meta-analysis to the two cohort studies that described the number of subjects who were smokers at baseline and later developed AD, they found smoking almost doubled the risk of AD (RR 1.99, 95% CI 1.33–2.98) (3). In accord with these data, this group also showed smoking was positively associated with decreased gray matter density in brain regions previously associated with incipient AD (2).

HOW SMOKING MAY PROMOTE AD AT THE MOLECULAR AND CELLULAR LEVEL

Unlike pure nicotine, which we and others have found to be neuroprotective due to its activation of α -7 nicotinic acetylcholine receptors and reduction of proinflammatory signaling (18,58), cigarette smoke is an admixture of 4,000 different compounds (16,49). Of these, 81 have been classified as pharmacologically active, toxic, mutagenic, and carcinogenic in nature (42). Thus, it is not surprising that inhalation of this smoke would be toxic to the brain as well. The importance of highlighting cigarette smoking as a risk factor for AD is twofold in that it is modifiable (13), and also because it points to a need to understand how cigarette smoke contents can mechanistically promote this disease outcome, as cigarette smoking continues to be prevalent worldwide.

In this regard, we have attempted to begin to understand this phenomenon in vitro. First, a brief discussion of the molecular origin of A β , the central pathology of AD, will be reviewed. A β peptide generation and aggregation as plaques are key pathological events in the development of AD (9,15,18,24,41,45). A β peptides and plaques have been extensively studied and evidenced to be neurotoxic, as they are reported mediators of apoptosis (27,31), inflammation (6), and oxidative stress (23,34). For this reason, some of the earliest proposed therapeutic strategies focused on prevention or elimination of these A β peptides and subsequent deposits. A β peptides are produced via the amyloidogenic pathway of amyloid precursor protein (APP) proteolysis, which involves the actions of β - and γ -secretases (43,46). Initially, β -secretase (BACE) cleaves APP, creating an A β -containing carboxyl terminal fragment known as β -C-terminal fragment (β -CTF) (48). This proteolysis also generates an amino terminal, soluble APP- β (sAPP- β) fragment, which is released extracellularly. Intra-cellularly, β -CTF is then cleaved by a multiprotein γ -secretase complex that results in generation of the A β peptide and a smaller γ -CTF (11,50). Oligomeric A β species are thought to be a driving force in AD-type neurodegeneration (54). A β peptides are metastable and can exist as monomeric, dimeric, and higher molecular weight oligomeric forms both in vitro and in vivo (54). It is becoming clear that A β dimers and oligomers are likely the neurotoxic species, because direct in vivo administration of these A β conformers injures neurons (54).

As cigarette smoke is an admixture of 4,000 different compounds (16), we used cigarette smoke condensate (CSC) to measure its effects in vitro. Since cigarette smoke is known to promote oxidative stress (32), which itself is known to promote β -secretase APP cleavage (51,57), we hypothesized that smoking may increase AD risk at the molecular level by activating this enzyme. To examine the effect CSC has on APP proteolysis, we first treated SweAPP N2a cells (N2a cells transfected with the human “Swedish” mutant form of APP) with a range of concentrations. Following Western blot, and ELISA analyses, we found that CSC effectively increases both A β _{1–40}, 42 production in a concentration-dependent manner (Fig. 1).

Microglia are activated in close vicinity of A β plaques in AD patient brains and in transgenic mouse models of the disease (6). To determine the extent to which CSC promotes a proinflammatory phenotype of microglia, we exposed BV2 microglial cells to CSC at various concentrations for 24 h. Compared to control, CSC at doses of 20 μ g/ml significantly promoted TNF- α and IL-1 β release ELISA (Fig. 2).

Next, since cigarette smoking has been positively associated with increased brain deposition of A β / β -amyloid and AD-like pathology (2,52) and microglial phagocytosis of A β has been considered a principle mechanism for the removal of A β from the brain parenchyma (4), we evaluated whether CSC could inhibit microglial uptake of A β . “Aged,” FITC-tagged A β _{1–42}

was added to primary cultured microglia for 60 min in the presence of varying concentrations of CSC or PBS (negative control). As shown in Figure 2, addition of CSC significantly and dose-dependently decreased microglial uptake of A β _{1–42} compared with PBS (negative control; $p < 0.05$) for each of the doses of CSC tested (Fig. 3).

In sum, the results of previous studies indicating smoking may be protective for, or not inducing, AD are not supportable, especially in light of the most recent, well-designed, large clinical studies by ourselves and others which clearly indicate that moderate to heavy smoking is a significant direct risk factor for AD (8,25,28,35,36,39,40). Currently, Reitz and colleagues (38) are developing an algorithm for generating a summary risk score for prediction of AD development in the elderly. Interestingly, in the resulting scale, the risk factor of smoking aided in predicating development of late onset AD will be included (38). Taken together, moderate to heavy smoking is associated with increase of AD. The mechanism underlying this phenomenon may involve the effect of cigarette smoke on APP processing, a reduction of A β clearance by microglia, and/or an increased proinflammatory profile initiated or perpetuated by microglia. Future in vivo studies will most certainly be required to fully uncover the mechanisms by which cigarette smoke may promote AD at the cellular and molecular level.

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Biographies

Dr. Brian Giunta received his Bachelors in Microbiology at the University of South Florida in 1998. He earned his M.D. at the University Of South Florida College of Medicine in 2004. Dr. Giunta then completed his internship in Psychiatry at Duke University Medical Center in Durham, NC in 2005. Following, he earned his M.S. and Ph.D. in Medical Science from the University Of South Florida College Of Medicine in 2008 and 2010, respectively. Dr. Giunta became Assistant Professor of Psychiatry at USF in 2007 and is the recipient of an NIH Clinical Scientist Development award.

Dr. Juan Deng received her M.S. and M.D. from the Third Military Medical University of China, Chongqing in 2001 and 2009, respectively. She completed a research fellowship in Neurology from 2004 to 2010 also at the Third Military Medical University and has been an attending neurologist there since 2006.

Dr. Jingji Jin is a senior postdoctoral fellow in the Department of Psychiatry and Neurosciences College of Medicine at University of South Florida. She received her B.A. from Dalian University of Technology, and the M.S. from Dalian University of Technology in China. She earned her Ph.D. from the Ehime University School of Medicine in Japan, and pursued her postdoctoral research in the Departments of Cancer Biology and Pharmacology at University of Illinois College of Medicine prior to joining Dr. Giunta's laboratory.

Mr. Edin Sadic is a third year undergraduate student at University of South Florida. He is currently studying BioMedical Sciences at USF and is following the Honors College 7-year Medical Program. Mr. Sadic be starting medical school at the University of South Florida College of Medicine in Fall 2012. He has spent the last 2 years gaining research experience in the lab of Dr. Giunta.

Ms. Saja Rum is an undergraduate student at the University of South Florida currently in her fourth year majoring in Biomedical Science. After graduating in the spring of 2012, Ms. Rum will obtain her Master's in Physician Assistant studies.

Dr. Huadong Zhou received his M.D. and Ph.D. from the Third Military Medical University, Chongqing, China in 1981 and 1994, respectively. He completed clinical and research fellowships at Flinders University of South Australia in 2003 and at St Luke's Brain and Stroke Institute in Kansas City, USA, in 2007. Currently, he is Chairman of the Department of Neurology at Third Military Medical University affiliated with Daping Hospital, Chongqing, China. Dr. Zhou specializes in diagnosis and treatment of Alzheimer's disease, stroke, cerebrovascular interventional treatment, and sleep disorders. Dr. Zhou's publications have focused heavily on epidemiological risk factors for stroke and dementia.

Dr. Paul R. Sanberg is Senior Associate Vice President for Research & Innovation, Distinguished University Professor, Executive Director of the Center of Excellence for Aging and Brain Repair, and Vice Chairman of Academics for the Department of Neurosurgery and Brain Repair in the College of Medicine, at the University of South Florida. Dr. Sanberg trained at York University, the University of British Columbia, the Australian National University, and Johns Hopkins University School of Medicine, among others. Dr. Sanberg is an inventor on approximately 30 health-related patents issued by the U.S. Patent and Trademark Office (USPTO), and numerous foreign patents. His research has focused on discovering innovative ways to repair damaged brain, and has helped lead the team that demonstrated that bone marrow and umbilical cord blood-derived stem cells can be transformed to neural cells that may be useful in stroke, spinal cord injury, and ALS. Dr. Sanberg is the founder of the National Academy of Inventors (at the University of South Florida) and currently serves as its president.

Dr. Jun Tan received his Bachelors of Medicine in China in 1983. He earned his M.S. at Fudan University in 1989 and Ph.D. in 1992, as well as postdoctoral studies at the University of Michigan in 1998. He became Assistant Professor of Psychiatry at USF, Associate Professor in 2004, and Professor in 2007. He is the author of 110 original scientific papers in prestigious journals such as *Science*, *Nature Neuroscience*, *Nature Medicine*, *Nature Communications*, *EMBO Journal*, and *Proceedings of the National Academy*

of Sciences. He is the recipient of multiple grants from the NIH totaling \$10 million. In 2007, Dr. Tan was named the Robert A. Silver Endowed Chair in Developmental Neurobiology.

Figure 1.

Cigarette smoke condensate (CSC) promotes Aβ generation in cultured neuronal cells. Aβ species were analyzed in cell lysates from CSC-treated SweAPP N2a cells by ELISA ($n = 3$ for each condition). Data are represented as the mean $\pm$ SD of a percentage of Aβ peptides secreted 24 h after CSC (Murty Pharmaceuticals, Lexington, KY; the batch of CSC is prepared from University of Kentucky reference cigarettes K1RF) (33,49) administration versus PBS. One-way ANOVA followed by post hoc comparison revealed significant differences between CSC-treated cells compared to control at 20 and 50 μ g/ml treatment concentrations ($p < 0.001$ and $p < 0.05$, respectively).

Figure 2.

CSC increases proinflammatory cytokine release in BV2 microglia. Mouse microglial cells were plated in 12-well tissue culture plates (NuncTM, Roskilde, Denmark) at 4×10^5 cells/well in DMEM media with 10% FBS and antibiotics. Cells were treated with CSC at various concentrations as indicated (from 5 to 50 $\mu\text{g/ml}$) for 24 h. Cell-free supernatants were then collected and assayed by TNF- α and IL-1 β ELISA kits (eBioscience, San Diego, CA) in strict accordance with the manufacturer's instructions. Compared with control, CSC treatment significantly increased TNF- α and IL-1 β release at doses of 20 $\mu\text{g/ml}$ and more.

* $p < 0.05$; ** $p < 0.01$ (see Fig. 1) by one-way ANOVA statistic analysis.

Figure 3.

CSC inhibits microglial phagocytosis of A β peptide. To determine whether CSC impairs microglia phagocytic capacity, “aged,” FITC-tagged A β ₁₋₄₂ was added to primary cultured BV2 microglia for 60 min in the presence of varying concentrations of CSC or PBS (negative control). As shown, addition of CSC significantly and dose-dependently decreased microglial uptake of A β ₁₋₄₂ compared to PBS (negative control; $p < 0.05$) for each of the doses of CSC tested except the 5 μ g/ ml dose.