

**Effects of apolipoprotein E polymorphism on the development of stroke**

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**Introduction**

Stroke is the third leading cause of death in the U.S. and the second most frequent specific determinant of age adjusted death.<sup>1</sup> As stroke more often results in disability than death, the overall loss in terms of person years, DALY, resources and energy is such that it has become one of the leading health issues all over the world.<sup>1</sup>

Several classical risk factors for stroke such as hypertension, alcohol consumption and cigarette smoking have been ascertained and established on firm ground of evidence through their pathophysiological correlation with etiology of stroke.<sup>1</sup> Association studies done in different populations all over the world have controversial results about the effect of serum cholesterol level in the etiology of stroke. In western populations, a positive association between serum cholesterol levels and the risk of stroke has been noted, whereas an inverse relationship between serum cholesterol and occurrence of intracerebral hemorrhage (ICH) and Cerebral Infarction has been observed in certain Asian populations.<sup>2</sup>

Lipids form an important structural and functional part of the human body. A normal healthy individual takes about 90 to 150g of lipids in his diet daily. Although this seems to be a large quantity, the concentration of lipids in the blood is closely regulated. Any disturbance in the regulation of lipids, especially of the cholesterol esters, can lead to a range of vascular and related disorders. Most prominent among these disorders is atherosclerosis. Apart from itself being a dangerous vascular change, atherosclerosis can lead to a variety of disabling and fatal conditions, depending on its site of origin. One of its secondary complications is stroke.

Cholesterol is the most abundant sterol in the body. Apart from dietary cholesterol, virtually all the tissues of body synthesize cholesterol, with liver, intestine, adrenal cortex and reproductive organs being the main contributors to the endogenous

cholesterol pool.

Cholesterol is an essential component of all natural membranes, which is its most important function the body. Liver is the main metabolic organ involved with cholesterol regulation. It receives cholesterol from three sources: a) dietary cholesterol, b) de novo synthesis and c) synthesis in extra hepatic tissues.

As cholesterol is insoluble in aqueous solutions, its transport takes place in the form of particles, which are rendered soluble by the presence of apolipoproteins on their surface. The apolipoproteins and polar lipids form a non-molecular film that surrounds the non-polar lipids in the core of the particle. The apolipoproteins (APO) involved are APOB48, APOB100, APOCII and APOE. If the metabolism and production of any of these lipoproteins is disturbed, it will lead to derangements in cholesterol metabolism leading to either hypercholesterolemia or hypocholesterolemia, which are important risk factors for many disorders including IHD, hypertension and hepatic disorders.

Shore and Shore<sup>3</sup>, first identified APOE as a constituent of very low density lipoprotein (VLDL) in 1973. Initially it was termed as the "arginine-rich lipoprotein." Later, a relationship between plasma distribution of APOE and dietary cholesterol level was also discovered. Experimental animal studies revealed that APOE becomes a part of the cholesterol-enriched particles that accumulate in plasma of animals, fed high levels of fats and cholesterol. We now know that these cholesterol-rich lipid particles are chylomicron remnants, VLDL and HDL, and that APOE is involved in their metabolism.

Development in the techniques of molecular biology has brought genes under perspective, as the root cause of virtually every disease known to Man. Much work has been done all over the world to find any association between the genetic variants of apolipoproteins and risk of stroke. In this regard, the most studied apolipoproteins have been APOB, APOCII and APOE. Apart from these, angiotensin-converting enzyme (ACE), paraoxonase (PON), G-protein and renin genes have also been studied for their association with atherosclerosis and stroke.

APOE has attracted considerable attention due to the controversial results of different studies done all over the world. The APOE gene has been found to be one of the factors contributing to inter-individual variations in serum cholesterol levels. The genetic variation in the APOE gene explains about 7% of the variations in total cholesterol levels in Caucasian population.<sup>4</sup>

This review focuses on the structure and function of APOE and the effects of its genetic variations, in order to put the possible roles of APOE in stroke into perspective.

## **APOE Gene**

APOE is present on chromosome 19q13.2.5 It is 3.7 kilobase pairs long and consists of four exons and three introns.<sup>6</sup> A promoter sequence TATAATT is present approximately 30bp upstream from the transcriptional initiation site. Other enhancer and promoter elements have also been found which can affect its biosynthesis.<sup>6</sup> APOE is linked to APOC1 and APOC1 pseudo-genes that are present in its vicinity on the chromosome.<sup>7</sup> Complete functional mapping of the human genome may reveal other factors and genes that possibly affect the expression of APOE or are in linkage disequilibrium with it.

APOE mRNA is 1163 bp in length.<sup>7</sup> The study of messenger RNAs has been a useful resource for scientists to evaluate tissue-specific syntheses of different proteins. APOE mRNA has been found in all organs with its larger concentrations in the liver, spleen, adrenal gland and brain.<sup>8</sup> An important exception is the intestinal epithelium which otherwise is actively involved in dietary lipid and cholesterol uptake. Liver is a major source of APOE and accounts for about two thirds of the plasma APOE concentration. Hepatic parenchymal cells are primarily responsible for its synthesis. In the brain, which is the second most active organ in APOE production, astrocytes are the principal cell type involved in its synthesis.<sup>9</sup>

## **Protein Synthesis and Structure**

The APOE mRNA is translated into a 317 amino acid product. The first 18 amino acids on the amino terminal of this nascent peptide, serve as a signal sequence that is later cleaved off.<sup>7</sup> The mature APOE is a 34.2 KD product<sup>10</sup>, which is secreted from the cells in combination with phospholipids and occur as APOE-phospholipid disks that have an approximate density of 1.08g/.<sup>8</sup>

APOE is composed of two main structural domains that probably also serve as its functional domains, an amino terminal domain that includes the LDL receptor-binding site and a carboxy terminal domain that has high affinity for lipids. Digestion of APOE

with thrombin yields a 22 kD fragment and a 10 kD fragment corresponding to the amino and carboxy terminal domains respectively.<sup>8</sup>

Experimental studies have shown that amino acids 1-183 are sufficient for normal APOE binding to the LDL receptor.<sup>11</sup> Detailed analysis has shown this amino terminal domain be composed of a four helix bundle that constitutes the receptor binding site. This region contains many basic amino acids that are thought to interact with the acidic residues in the ligand-binding domain of the LDL receptor.<sup>12</sup> The carboxy-terminal domain, which consists of amino acids 216-299, is thought to be the lipid-binding domain. This region is a strong amphipathic  $\alpha$ -helical structure that has a polar side and a lipid-binding non-polar side.<sup>8</sup>

## **Physiological Role of APOE**

APOE is one of almost more than ten protein constituents of plasma lipoproteins that are involved in various metabolic and transport pathways.<sup>8</sup> APOE possibly has multiple roles in the body, one of which is its function in maintenance of lipoprotein particles and lipid transport. APOE is also thought to be involved in nerve growth<sup>9</sup> and regeneration<sup>13</sup>, which may explain its high concentration in the nervous tissue and CSF.<sup>14</sup> APOE also has a "paracrine like" mode of action. It is involved in movement of cholesterol from cholesterol rich cells to those with its lesser concentration, within an organ or tissue. This accounts for the large concentration of APOE in the interstitial fluid. We will restrict our discussion to its involvement in lipid metabolism.

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## APOE Variants

To date, about 30 APOE variants have been characterized.<sup>15-17</sup> The most common APOE variants in different populations all over the world are APOE  $\epsilon$ 2, APOE  $\epsilon$ 3 and APOE  $\epsilon$ 4. All other variants have been designated according to their positions, relative to these three after isoelectric focusing.<sup>18</sup> APOE<sub>2</sub>, E<sub>3</sub> and E<sub>4</sub> differ at a single locus in the gene. These alleles differ by base substitutions at codons 112 and 158 (Figure). APOE  $\epsilon$ 3 has not been associated with any disorder and therefore taken as the reference allele in all studies. Effects of APOE Polymorphisms on Protein Function

As discussed earlier, APOE has three major variants that are the most common APOE alleles in the existing human population. The mature protein products of these three alleles differ at two amino acid positions. APOE  $\epsilon$ 3 has a cysteine residue at position 112 and an arginine residue at position 158 while APOE  $\epsilon$ 2 has cysteine and APOE  $\epsilon$ 4 has arginine residues at both these positions.

APOE  $\epsilon$ 3 is the most frequent allele in all populations and age groups with a frequency of

over 60%.<sup>4</sup> Although it is not thought to be the ancestral allele, any involvement of APOE  $\epsilon$ 3 in any disorder has never been observed. APOE  $\epsilon$ 3 binds to its receptor normally and there

[(0)]

is no evidence of any association between APOE  $\epsilon$ 3 carrier status and lipid dysregulation.

APOE  $\epsilon$ 2 has a significantly reduced affinity for its receptor as compared to APOE  $\epsilon$ 3.<sup>4</sup> In this case, the substitution of arginine with cysteine at position 158 is thought to disturb the conformation of the  $\alpha$ -helical structure of this part of the protein, which reduces its ability to bind to the receptor.<sup>4</sup> Arginine being a basic amino acid probably serves to enhance the affinity of the protein for its receptor that has acidic residues in its ligand-binding domain.

The hepatic uptake of HDL and VLDL remnants, IDL and HDL is dependant upon binding of APOE to its receptor. Due to lower affinity of APOE  $\epsilon$ 2 for its receptor, the uptake of these particles decreases. To compensate for the reduced uptake of cholesterol and to control serum cholesterol level, the hepatocytes upregulate LDL receptors on their membranes.<sup>19</sup> As a result, LDL decreases in blood by its APOB-100 mediated uptake. Hence, dysbetalipoproteinemia is a common finding in  $\epsilon$ 2 homozygotes. These individuals have elevated plasma levels of VLDL triglycerides, VLDL cholesterol and chylomicron remnants, while LDL and HDL are usually low. There is an increased risk of atherosclerosis and its complications in these people.

APOE  $\epsilon$ 4 binds to LDL receptor normally but it attaches to the dietary derived lipoproteins and VLDL more readily as compared to APOE  $\epsilon$ 3.<sup>8</sup> Crystal structure analysis of APOE  $\epsilon$ 3 suggests that arginine at position 112 (as in APO E<sub>4</sub>) may disrupt specific interactions between the amino and carboxy terminals of the protein and hence alter its affinity for lipids. As a result, there is an increased clearance of these particles by the hepatocytes. The resultant decrease in total plasma cholesterol induces the hepatocytes to downregulate APOB-100 LDL receptors.<sup>16</sup> Thus,  $\epsilon$ 4 carriers have higher LDL cholesterol levels in blood as compared to  $\epsilon$ 3 carriers. Increased LDL cholesterol puts them at a high risk of atherosclerosis.

APOE  $\epsilon$ 2 homozygotes have a reduced LDL cholesterol level as compared to  $\epsilon$ 3 and  $\epsilon$ 4 homozygotes; hence, they may be at a lower risk for atherosclerosis and ischemic stroke. Cholesterol being an important constituent of cell membranes, the  $\epsilon$ 2 carriers have a greater chance of endothelial weakening in intracerebral arteries because of their low cholesterol levels.<sup>20</sup> In some populations, APOE  $\epsilon$ 2 homozygotes have been shown to have a significantly higher risk of getting intracerebral hemorrhage.<sup>21</sup> On the other hand, increased LDL cholesterol in  $\epsilon$ 4 carriers increases their risk of developing atherosclerotic vascular disease. Higher plasma cholesterol levels may enhance the formation of

atheromatous plaques in the fatty streaks. Hence, these people are a high risk group for ischemic stroke. Atherothrombotic process may add to the effects of underlying atherosclerosis.<sup>22</sup>

## Prevalence of Stroke

Cerebrovascular disease is the second leading cause of death, worldwide.<sup>23</sup> It is the leading cause of DALYs lost among persons aged 60 and above. The incidence of stroke varies in different parts of the world. Epidemiology of stroke had been extensively studied in Europe, North America and Eastern Asian countries. Unfortunately, most of the data available from these and other countries shows the mortality of stroke instead of its incidence. Still the mortality patterns of stroke are a good measure of its incidence, because there does not seem to be much difference in the two.

The strike rate of stroke, when compared internationally does not show any regular geographic pattern. Generally, China, Japan, Taiwan, Eastern European countries, Trinidad and Tobago and the former Russian states have some of the highest incidence rates in the world.<sup>24</sup> Stroke is the number one cause of death in China, Hungary and Brazil.<sup>25</sup> The lowest incidence rates are found among the affluent western European countries and North America. Canada, France, Switzerland and Australia also have some of the lowest incidence rates.<sup>24,25</sup>

During the four decades, from 1950 to 1990 there has been a decline in the incidence and mortality of stroke all over the world. This decline was more pronounced in Established Market Economy Countries (EMEC), which include the U.S, Japan, Europe, Finland, Sweden and Switzerland, among others. By far the largest declines in incidence have been observed in Japan and Finland.<sup>25</sup> Eastern European countries and China have improved, yet continue to be high incidence rate zones.<sup>25</sup> In the last decade, incidence rates have stabilized across the globe but the incidence is on the rise again in some countries.

In all the populations studied, men have higher incidence than women. The temporal trends in mortality are also more favorable in women than in men. Hormonal differences are thought to play a part on creating this gap, but the evidence is still conflicting.<sup>26</sup> Postmenopausal women are apparently at an almost equal risk of getting stroke, as compared to men.<sup>26</sup>

By far, the most important risk factors are age and lifestyle. The incidence of stroke increases a 100 folds from around 30/100 000 in the 3rd and 4th decades, to almost 300/100 000 in those aged 80 and above.<sup>1</sup> The risk of stroke increases by 80% with every ten years of life.<sup>1</sup> Race is also an

important non-modifiable risk factor, which puts blacks at a higher risk for stroke. Modifiable risk factors like diabetes mellitus, hypertension and lipid profile are the most important determinants in the etiology of stroke.

### Association of APOE polymorphism with Stroke

APOE holds a key position in the lipid metabolism in the body; hence, its role in the development of lipid related vascular diseases has been under perspective since it was discovered. APOE gene polymorphisms has been studied in different populations for putative roles in the development of stroke. Results have been controversial or not statistically significant, and no definitive conclusion has been reached (Table). Analysis of the important case control studies done so far, does not yield any conclusion as half of the results are insignificant and the remaining do not support each other.

According to Couderc et al<sup>27</sup>, APOE  $\epsilon$ 2 is associated with ischemic cerebrovascular disease (ICVD) in the French population. At the same time, APOE  $\epsilon$ 4 has been associated not only with ICVD<sup>19,28</sup> but also with large vessel ICVD. In the western populations, APOE was shown to be unrelated to cerebral infarction. In a south Asian population cerebral thrombosis was associated with APOE  $\epsilon$ 4 carrier status and APOE  $\epsilon$ 2 was found to be significantly present in hemorrhagic stroke patients.<sup>29</sup> In the stroke, morbidity and mortality region of Japan APOE was found to be unrelated to cerebral infarction as well as to ICH.

Age is an important factor in determining the role of APOE in the development of stroke. Some studies show a protective effect of APOE  $\epsilon$ 2 in older populations.<sup>30</sup> Others do not show any relationship at all. The majority of the case-control studies done so far, did not include a significantly large sample size to give power to their conclusion. If calculated separately, results would also

Table. Worldwide association studies of APOE alleles with stroke.

First author (published year) Reference No.	Studied event	Cases No. Controls No.	Average age	Allele %†			Association
				$\epsilon$ 2	$\epsilon$ 3	$\epsilon$ 4	
Chowdhury et al (2001)	stroke	322	67.9±11.0	9	79	12	$\epsilon$ 2-atherothrombosis, cardioembolism,
		1126	64.3±10.5	5	85	11	$\epsilon$ 4-atherothrombosis, ICH, SAH
Peng et al (1999)	ICVD	90	63	8	79	13	$\epsilon$ 3/ $\epsilon$ 4
		28	90	63	11	83	6
Margaglione et al (1998)	Survived ICVD	100	66	6	76	18	$\epsilon$ 4
		5	108	61	8	85	6
Nakata et al (1997)	Thrombosis	55	66	2	89	9	-
		61	67	6	90	4	-
Kessler et al (1997)	ICH	38	63	7	86	7	-
		227	62	9	76	15	-
Coria et al (1995)	ICVD	225	63	7	81	12	-
		104	71	6	82	12	-
Pedro-Botet et al (1992)	Survived ICVD	34	94	72	778†	78†	15
		69	72	7	85	9	$\epsilon$ 2
Mahieux et al (1990)	ICVD	100	64	8	73	19	$\epsilon$ 3/ $\epsilon$ 4
		19	100	64	8	82	11
18		59	73	684	84	10	-
		28	72	0	96	4	-

- indicates no association.

† in some cases, percentages may not sum up to 100% due to rounding up.

† only subjects with 73/73 were included.

differ in males and females due to difference in morbidity rates between the two genders.

These results suggest a polygenic basis of stroke pathogenesis. The gene-environment interaction may

have an important part in explaining the variations in the allelic associations found in different regions of the world.

#### Special Populations

In certain parts of the world, strange patterns of APOE allele distribution have been observed. Among them are the Amerindians, who have no APOE  $\epsilon$ 2 allele.<sup>31</sup> The same is the case of Mexican Mayans and Yanomas of Brazil. The frequency of APOE  $\epsilon$ 2 is either very low or nil in the Squimos of Greenland. Certain African Pygmy populations also have no APOE  $\epsilon$ 2 homozygotes in the population. At the same time, the proportion of APOE  $\epsilon$ 4 is very high in these individuals. APOE  $\epsilon$ 2 is also absent in the Tutsi of Burundi and the Fon from Benin. On the other side of the world, the Australian Aborigines of Mowanjum also appear to be devoid of APOE  $\epsilon$ 2 allele.<sup>32</sup> To date, no such population has been found where APOE  $\epsilon$ 4 allele is absent. These inter-ethnic variations may be geographical in origin, and close observation of these populations for development of vascular diseases and problems of lipid metabolism may help to improve our insight into the pathogenesis of cerebrovascular diseases.

### **Possible Role of APOE in development of stroke**

So far, the exact association of any APOE variant with stroke has not been established. The different possible mechanisms through which APOE has been proposed to act are probably only a few of its roles in the body. APOE has been hypothesized and in some cases shown to have several completely different roles in the body, which range from lipid metabolism to immunologic and neurogenerative actions.

It has been shown that in the presence of  $\beta$ -VLDL, APOE  $\epsilon$ 3 increases neurite outgrowth, whereas APOE  $\epsilon$ 4 decreases the outgrowth, in vitro studies.<sup>33</sup> Hence, APOE may be involved in the regeneration of lost neuronal tissue and this may be true for post-ischemic regeneration in the brain. Therefore, one particular variant of APOE may actually render a person stroke resistant while the other makes him stroke prone, even in mild hypoxic insults. This is supported by the fact that multiple sclerosis patients who are in the remyelination phase have elevated levels of APOE in the CSF.

APOE also has specific receptors on macrophages and oligodendrocytes.<sup>34</sup> APOE  $\epsilon$ 2 binds defectively to these receptors, so it may inhibit repair mechanisms and hence increase the parenchymal damage after cerebral ischemia. APOE may play a role in the development of atherosclerosis by affecting the growth, proliferation and differentiation of smooth muscle cells in the arterial walls.<sup>35</sup> All

these and other mechanisms may act synergistically or individually to cause or prevent cerebrovascular pathologies but no single factor has yet been found, the presence or absence of which can cause a cerebrovascular disease on its own. Stroke, like hypertension has a polygenic basis and may be the result of many indirectly acting factors. Hence finding out more and more intermediate phenotypes would help in reaching to its root cause.

We hypothesize that APOE variants may act in the pathogenesis of stroke by a mechanism mediated through atherosclerosis. Studies done to date demonstrate that the prevalence of APOE  $\epsilon$ 3 allele is high in both stroke patients and controls ranging from 73%-96% (Table). APOE  $\epsilon$ 3 being the normal variant, its high prevalence in both diseased and healthy populations hint us to the fact that APOE may be involved in the overall pathogenesis of stroke in not an extremely major way. As we are yet unaware of the boundaries of action of APOE, we can suspect any additional role that a genetic variant may take up. Complete functional mapping of the human genome will help in determination of any other genes and gene products that effect the action of APOE in the body. The discovery of the role of APOE in stroke will be of immense diagnostic importance and will help to decrease the physical, psychological and economic sufferings caused by stroke.

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