

## Special Article - Alzheimer's Disease

# Galactosylated N-Glycans in the Choroid Plexus: A Possible Aging-Related Molecular Therapeutic Strategy for Alzheimer Disease

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## Abstract

Choroid Plexus (CP) and endothelium have important physiological and pathological roles in Alzheimer Disease (AD). The AD triple transgenic mouse model (3xTg-AD) is an animal model that mimics many critical hallmarks of the disease. Considering aging as the main AD risk factor, the AAV9 vector efficiency to carry out a non-invasive gene delivery in the CP of aged mice was studied. The results showed a greatly reduced GFP expression in the CP of aged mice compared to young mice. To explore the possible mechanisms involved in the drastic reduction of GFP, possible AAV9 neutralizing factors were analysed in the mice-sera. There were no significant differences between young and old mice. The next step was to assess cell-surface N-linked glycans with terminal galactosyl residues since serve as the primary receptor for AAV9. We studied N-glycans in the CP of aged and 3xTg-AD mice by fluorescence staining using a biotinylated lectin and eNOS, a marker of endothelium. A decrease in terminally galactosylated N-glycans was also found in aged and 3xTg-AD mice. The results indicated that very reduced AAV9 tropism in the CP of aged mice correlates both with a significant reduction in galactose residues of glycans that occurs in aging, as well as with a decrease of eNOS in endothelial cells. In order to increase AAV9 tropism in CP both a neuraminidase-based molecular therapeutic strategy and an improvement of endothelium function for AD is proposed.

**Keywords:** AAV9; Aging; Alzheimer disease; Choroid plexus; Galactosylated N-glycans; Endothelium

## Introduction

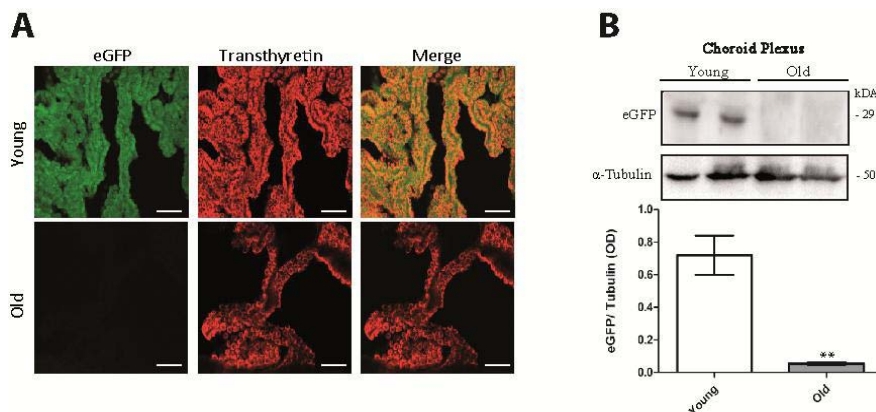
Alzheimer's Disease (AD) is the most common form of neurodegenerative dementia in the elderly. The pathological features that characterize AD are neuronal atrophy, synapse loss and the progressive accumulation of senile plaques. These plaques are composed of various Amyloid Beta (A $\beta$ ) peptides, including the 40 and 42 amino acid cleavage products (A $\beta_{40}$  and A $\beta_{42}$ ) of the amyloid precursor protein, and intracellular Neurofibrillary Tangles (NFTs), containing hyperphosphorylated tau protein [1,2]. This accumulation in AD is quite toxic for neurons mostly in cortex and hippocampus [3], however increasing data for animals support the notion that compromised function of Choroid Plexus (CP) and defective cerebrospinal fluid production and turnover with diminished clearance of the A $\beta$  peptides normally produced in brain, may be a mechanism implicated in the exacerbation of AD [4]. Additionally, it has been suggested that the accumulation of A $\beta$  in CP further increases even more the disruption of the blood-cerebrospinal fluid barrier [5,6], which may impact on neurodegeneration. Currently, little is known about transport and metabolic responses of CP to the disrupted homeostasis of A $\beta$  in AD.

Although CP participates in the development of AD, another important factor in the clearance of A $\beta$  is the vascular system. There are various authors describe that the increase of oxidative Reactive Oxygen Species (ROS) lead endothelial dysfunction both animal

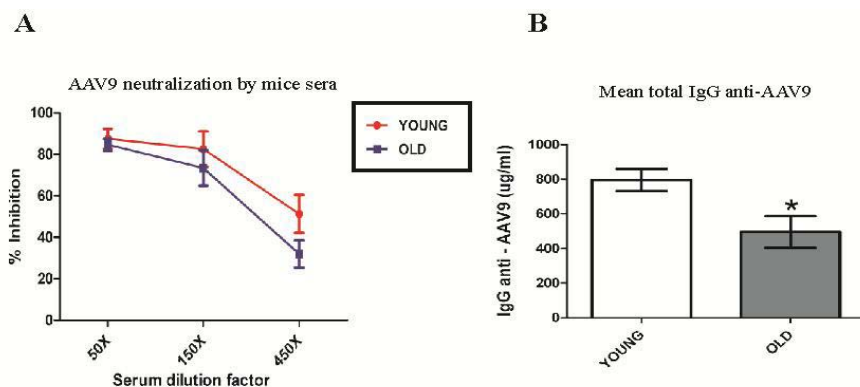
models [7,8] and humans [9,10]. Besides there are clear evidences that endothelial dysfunction is primarily responsible for the pathogenesis that underlies Alzheimer disease [11].

The AD triple-transgenic mouse model (3xTg-AD) at 16 month-old mimics critical hallmarks of the human disease: A $\beta$  plaques and neurofibrillary tangles with a temporal- and regional- specific profile [12]. However, it is little known how CP dysfunction in aging can upset illness. In order to prevent AD-like pathology, our major goal is to restore the CP dysfunction in 3xTg-AD mice through the use of gene therapy, using as vector the non-invasive adeno-associated virus serotype 9 (AAV9). AAV9 has a high tropism in CP epithelial cells from young mice [13], due to the large existence of primary receptors of AAV9, consisting in membrane glycans with terminal galactose [14]. The problem is that the most studies are performed in young models, and aging, the main risk factor in AD, is scarcely included in the studies of this disease. The CP also presents age-related changes [15,16]. Specifically, aged CP epithelial cells present a general atrophy, and consequently Cerebrospinal Fluid (CSF) production diminishes, but also clearance of CSF out of the brain is delayed [17]. Nonetheless there are few studies that combine both molecular pathologies of AD and aging, as well as very few gene delivery preclinical studies in old animals.

In this work we analysed both how aging may affect AAV9 tropism in CP and others brain regions, as well as possible underlying



**Figure 1:** AAV9-mediated eGFP expression was greatly reduced in epithelial cells of the CP in old mice respect to young mice. A. A representative confocal microscopy image of the CP stained with antibodies anti-eGFP (green) anti-transthyretin (red) and the merge from young mice (6 weeks old) and old mice (22 months old) is shown. Scale bars, 50µm. B. Western blot for eGFP was carried out for quantifying the protein levels. n = 4 animals per group; \*\*p<0.001 Old vs. Young mice (t-test two tailed).



**Figure 2:** Neutralizing factors do not explain the poor AAV9 transduction in CP from old mice. A. Percent inhibition of transduction of AAV9 neutralized with mice sera dilutions. No significant changes were detected between sera of young and old mice (n=5, sera of non-AAV9 treated animals were also included as control). B. ELISA-IgG values against AAV9 in serum of young and old mice are shown (n=5, sera of non-AAV9 treated animals were also included as control). Serum IgG levels of young and old mice are significantly different. \*p<0.05 (t-test two tailed).

mechanisms. In addition, we studied the involvement of such mechanisms in a triple transgenic mouse model of Alzheimer’s disease.

**Materials and Methods**

**Animals**

3xTg-AD mice harboring three mutant genes: beta-APP (APP<sup>swe</sup>), presenilin-1 (PS-1M146V) and tauP301L, and the corresponding wild type mice, were provided by Dr. Lydia Giménez-Llort (Autonomous University of Barcelona, Spain). Eight 3xTg-AD mice and eight non-transgenic control mice (Non-Tg), 16 month-old, were used. For studies of aging, we used 6-week-old and 22-month-old C57BL/6j male mice. All animal experiments were approved by the bioethical committee of the University of La Laguna (Reference # 091/010) and are in accordance with the European Communities Council Directive of 22 September 2010 (2010/63/EU) regarding the care and use of animals for experimental procedures.

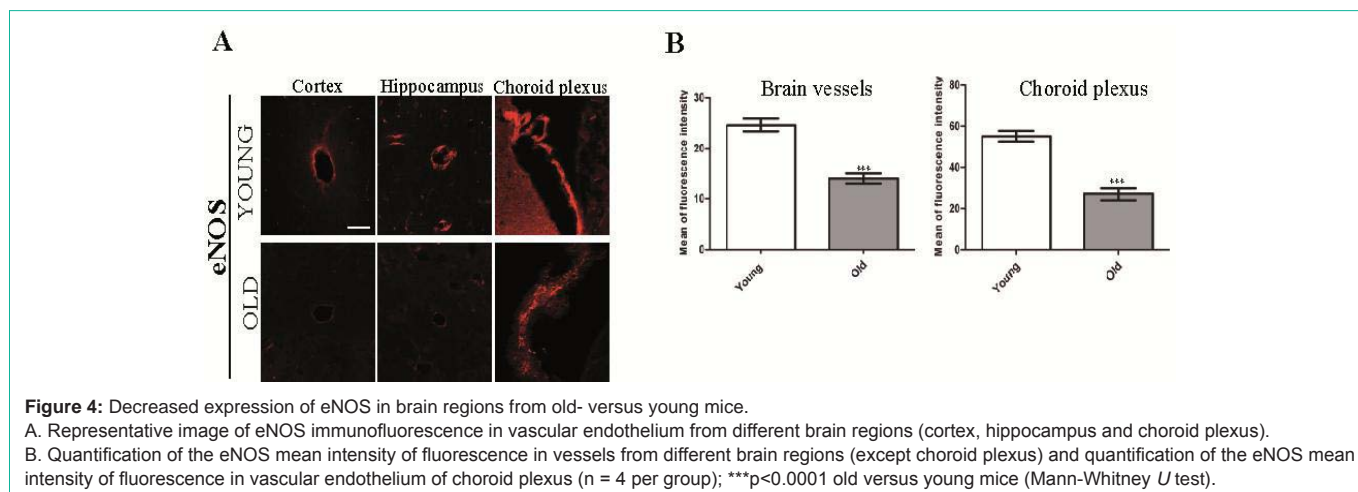
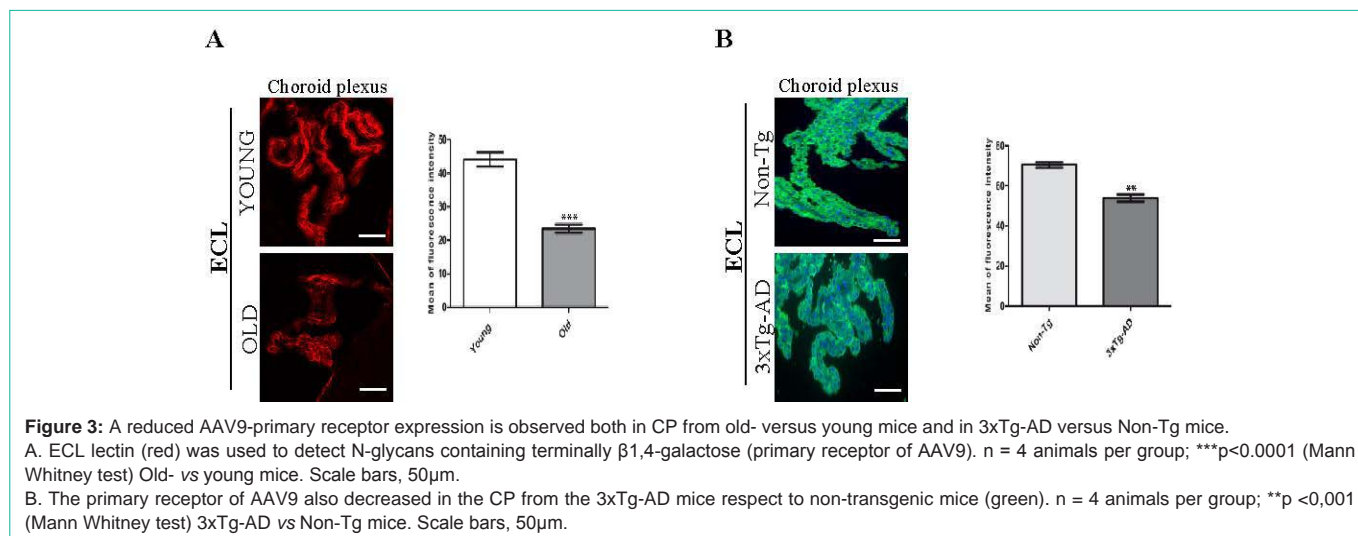
**AAV9-mediated gene delivery experiments**

Construction and production of adeno-associated vector

AAV9 vectors were synthesized at the Vector Production Unit of the Autonomous University of Barcelona using the triple transient transfection method with Polyethyleneimine (PEI). eGFP were used as reporter gene. HEK293 cells were harvested 72 hours after transfection and the viral particles were purified by ultracentrifugation on an iodixanol gradient according to the Zolotukhin et al. [18] method. We injected both groups of C57BL/6j mice with AAV9-eGFP intravenously at a dose of 1,3x10<sup>13</sup> viral genomes per kg in 100µl PBS (n=8 per group). This viral genome dose was chosen to avoid side effects as far as possible [18]. At 6 weeks post-injection mice were killed and subjected to either morphological analysis or molecular biology and biochemical studies.

**Western-blotting, Immunofluorescence and lectin-staining**

Proteins were isolated with M-PER extraction reagent (Pierce). Protein sample concentrations were equalized by the bicinchoninic acid method and separated by SDS-PAGE and transferred to a nitrocellulose membrane. The blotted membranes were incubated with anti-GFP antibodies (1: 50000; SySy). α-tubulin was used as



a loading control and Chemidoc-Quantity-One software (Biorad Laboratories) was used to perform quantitative analyses. For immunofluorescence, the sections of fixed tissue were incubated with anti-GFP antibody (1: 1500; SySy), eNOS (1:1000; Sigma) and anti-transthyretin (1:200; DAKO). For studying the primary receptor of AAV9 we used the Erythrina cristagalli lectin (ECL) whose binds to the galactose residues of cell-surface glycans [14]. Staining was performed using this biotinylated lectin ECL (Vector Laboratories, Burlingame, CA, USA) to 1:200. Lectin was visualized using Streptavidin-CY3 or Streptavidin-CY2 (Vector Lab, Burlingame, CA; 1:600). The fluorescence images were taken with a confocal microscope Olympus Fluoview FV10 (Olympus FV10-ASW2Q, Shinjuku, Tokyo, Japan).

**Neutralizing factors**

To test for the prevalence of neutralizing factors against AAV9 in the sera of the mice, AAV2/9-CMV-luciferase was incubated at 37°C for 30 minutes with non-heat-inactivated serum samples diluted in infection medium (DMEM+2% FBS+1% PenStrep) to 1:50, 1:150 and 1:450. Luciferase activity was detected by adding luciferin substrate (Pierce Firefly Luciferase Flash Assay kit) and reading the resulting luminescence in VICTOR 3 (Perkin Elmer). Total concentration

of IgG antibodies against AAV9 present in the animal sera was determined by ELISA.

**Statistical analysis**

Data were reported as Means  $\pm$  Standard Error (SEM).Statistical comparisons were performed using unpaired two-tailed Student’s t-tests or Mann-Whitney U-test. Differences were considered to be significant when P $\leq$ 0.05.

**Results and Discussion**

Our study supports the ability of AAV9 to successfully traverse the vasculature and efficiently transducing CP of young mice, after intravenous administration of a marker gene (eGFP). However, we have found that this capacity decreases dramatically in several brain regions in 22 months old mice: Regarding CP, after performing immunofluorescence of eGFP and transthyretin, we observed a drastic reduction in the expression of eGFP in old mice compared to young mice (Figure 1A). These data were corroborated with the studies of immunoblotting where we also observed a reduction of eGFP levels in CP from old mice compared to young mice (Figure 1B).We found also a highly reduction of eGFP levels in cortex and hippocampus from old- compared to young mice (Supplementary

Figure 1).

The much reduced eGFP expression could be due to AAV9 neutralizing factors in serum of aged mice [19]. To ensure that AAV9 there arrived to CP, we evaluated both the neutralizing antibodies and other possible neutralizing factors in mouse sera which could prevent the arrival of AAV9 to CP. However, there were no significant changes both in the neutralizing factors (Figure 2A) as in IgG against AAV9, and even IgG was significantly lower in old-mice serum than in young mice (Figure 2B). This phenomenon could be due to the immunosenescence described as age advances in all species [20]. In mice, the strongest immune system is detected between 6 weeks and 6 months, while at 23 months the robustness of the immune response is much lower. However, we cannot discard the hypothesis that lower neutralizing factors and IgG titers in old animals could be due to the reduced level of transduction in this group of animals, leading to significantly less antigen-presenting cells and thus, to poorer activation of the immune system.

Once discarded neutralizing factors or possible antibodies against AAV9, since there was not statistical differences between serum from young- and old mice (Figure 2), the next step was to study the primary receptor of AAV9 in CP. ECL staining revealed that the terminal galactosylation of cell surface glycans was significantly reduced in CP from old mice compared to young (Figure 3A), it suggesting one possible mechanism of reduced efficiency transduction of AAV9 in CP from old mice. Moreover, ECL staining showed that the terminal galactosylation of cell surface glycans was also reduced in CP of 3xTg-AD mice respect to Non-Tg mice (Figure 3B). This could be a drawback for a future AAV9-mediated gene therapy targeted choroid plexus of 3xTg-AD mice. It has been described a correlation between agalactosylated IgG and aging human, and also in old-mice models [21]. Our data confirm this change of glycan profile, but in a very important structure for AD as CP. Many researchers think that the agalactosylated glycans are a good marker of aging; even in serum of patients with dementia these molecules are found a significant lower levels than controls [22], which could also be an excellent pathologic marker for AD.

Nevertheless, according to our results, the primary receptor amount only was 50% reduced in the old mice so that other factors could be influencing in the poor entrance of AAV9. Since the blood flow is lower in aging and Alzheimer disease [23], next step was to analyze eNOS immunofluorescence in different vessels of cortex, hippocampus and the CP. We found a significant reduction of eNOS in old mice respect young's (Figure 4). These data support that there is a reduction in blood flow from old mice, which together to the fall of the primary receptor could explain the almost null tropism of AAV9. However, these facts, which a priori could be negative, open a range of possibilities to improve the AAV9 tropism. One of them, proposed by Bell et al. (2011), consisted in using Neuraminidase (NA) to increase the abundance of the receptor on target cells, improving both the vector efficacy and delivering of AAV vectors to their therapeutic targets [24].  $\beta$ -1,4galactose is the commonly observed penultimate monosaccharide on most sialic acid-rich glycans [24], and NA is able to remove the sialic acid, and display the primary receptor of AAV9. However, it is not clear the role of both sialic acid-glycans as galactose-terminal glycans on aging and it would be advisable to

perform more studies to know how it would affect the use of NA at a systemic level. On the other hand, one of the best restorers of the vascular system is the physical exercise, which produces angiogenesis independent of aging [25,26]. The increase of blood vessel might enhance the amount of viral particles of AAV9 that arrive at the CP, and improve the tropism in AD.

Therefore, we suggest that both a neuraminidase-based molecular therapeutic strategy as an increase the angiogenesis through physical exercise might increase the AAV9 tropism in the CP for AD. These results may help to gain further insight in using AAV9 to repair the CP dysfunction described in 3xTg-AD mice.

## Conclusion

An important challenge is to find ways to selectively preventing or minimize CP damage and CSF functions both in aging and in states of neurodegeneration. Our results strongly suggest that AAV9 may be targeted noninvasively to CP, by using promoters of genes selectively expressed, to restore its function in AD.

## References

- Karran E, Mercken M, De Strooper B. The amyloid cascade hypothesis for Alzheimer's disease: an appraisal for the development of therapeutics. *Nat Rev Drug Discov.* 2011; 10: 698-712.
- Thinakaran G, Koo EH. Amyloid precursor protein trafficking, processing and function. *J. Biol. Chem.* 2008; 283: 29615-29619.
- Gomez-Isla T, Price JL, McKeel DW, Morris JC, Growdon JH, Hyman BT. Profound loss of layer II entorhinal cortex neurons occurs in very mild Alzheimer's disease. *J Neurosci.* 1996; 16: 4491-4500.
- Johanson CE, Duncan JA 3rd, Klinge PM, Brinker T, Stopa EG, Silverberg GD. Multiplicity of cerebrospinal fluid functions: New challenges in health and disease. *Cerebrospinal Fluid Res.* 2008; 5: 1-32.
- Vargas T, Ugalde C, Spuch C, Antequera D, Morán MJ, Martín MA, et al. Abeta accumulation in choroid plexus is associated with mitochondrial-induced apoptosis. *Neurobiol Aging.* 2010; 31: 1569-1581.
- Silverberg GD, Miller MC, Messier AA, Majmudar S, Machan JT, Donahue JE, et al. Amyloid deposition and influx transporter expression at the blood-brain barrier increase in normal aging. *J Neuropathol.* 2010; 69: 98-108.
- Csiszar A, Labinsky N, Orosz Z, Xiangmin Z, Buffenstein R, Ungvari Z. Vascular aging in the longest-living rodent, the naked mole-rat. *Am J Physiol.* 2007; 293: 919-927.
- Ungvari Z, Orosz Z, Labinsky N, Rivera A, Xiangmin Z, Smith K, et al. Increased mitochondrial H<sub>2</sub>O<sub>2</sub> production promotes endothelial NF- $\kappa$ B activation in aged rat arteries. *Am J Physiol Heart Circ Physiol.* 2007; 293: 37-47.
- Donato AJ, Eskurza I, Silver AE, Levy AS, Pierce GL, Gates PE, et al. Direct evidence of endothelial oxidative stress with aging in humans: relation to impaired endothelium dependent dilation and upregulation of nuclear factor- $\kappa$ B. *Circ Res.* 2007; 100: 1659-1666.
- Jablonski KL, Seals DR, Eskurza I, Monahan KD, Donato AJ. High dose ascorbic acid infusion abolishes chronic vasoconstriction and restores resting leg blood flow in healthy older men. *J Appl Physiol.* 2007; 103: 1715-1721.
- Aliev G, Palacios HH, Gasimov E, Obrenovich ME, Morales L, Leszek J, et al. Oxidative Stress Induced Mitochondrial Failure and Vascular Hypoperfusion as a Key Initiator for the Development of Alzheimer Disease. *Pharmaceuticals (Basel).* 2010; 3: 158-187.
- González-Marrero I, Giménez-Llort L, Johanson CE, Carmona-Calero EM, Castañeyra-Ruiz L, Brito-Armas JM, et al. Choroid plexus dysfunction impairs beta-amyloid clearance in a triple transgenic mouse model of Alzheimer's disease. *Front Cell Neurosci.* 2015; 9: 1-17.



13. Schuster DJ, Dykstra JA, Riedl MS, Kitto KF, Belur L, McIvor RS, et al. Biodistribution of adeno-associated virus serotype 9 (AAV9) vector after intrathecal and intravenous delivery in mouse. *Front Neuroanat.* 2014; 8: 1-14.
14. Shen S, Bryant KD, Brown SM, Randell SH, Asokan A. Terminal N-linked galactose is the primary receptor for adeno-associated virus 9. *J Biol Chem.* 2011; 286: 13532-13540.
15. Serot JM, Béné MC, Faure GC. Choroid plexus, aging of the brain, and Alzheimer's disease. *Front Biosci.* 2003; 8: 515-521.
16. Liu CB, Wang R, Dong MW, Gao XR, Yu F. Amyloid-beta transporter expression at the choroid plexus in normal aging: the possibility of reduced resistance to oxidative stress insults. *Sheng Li XueBao.* 2014; 66: 158-168.
17. Marques F, Sousa JC, Sousa N, Palha JA. Blood-brain-barriers in aging and in Alzheimer's disease. *Mol. Neurodegener.* 2013; 8: 1-9.
18. Zolotukhin S, Byrne BJ, Mason E, Zolotukhin I, Potter M, Chesnut K, et al. Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield. *Gene Ther.* 1999; 6: 1-14.
19. Gray SJ, Matagne V, Bachaboina L, Yadav S, Ojeda SR, Samulski RJ. Preclinical differences of intravascular AAV9 delivery to neurons and glia: a comparative study of adult mice and nonhuman primates. *Mol Ther.* 2011; 19: 1058-1069.
20. Manno CS, Pierce GF, Arruda VR, Glader B, Ragni M, Rasko JJ, et al. Successful transduction of liver in hemophilia by AAV-factor IX and limitations imposed by the host immune response. *Nat Med.* 2006; 12: 342-347.
21. Dall'Olio F, Vanhooren V, Chen CC, Slagboom PE, Wuhler M, Franceschi C. N-glycomic biomarkers of biological aging and longevity: a link with inflammaging. *Ageing Res Rev.* 2013; 12: 685-698.
22. Vanhooren V, Dewaele S, Libert C, Engelborghs S, De Deyn PP, Toussaint O, et al. Serum N-glycan profile shift during human ageing. *Exp Gerontol.* 2010; 45: 738-743.
23. Eberling JL, Jagust WJ, Reed BR and Baker MG. Reduced temporal lobe blood flow in Alzheimer's disease. *Neurobiol. Aging.* 1992; 13: 483-491.
24. Bell CL, Vandenberghe LH, Bell P, Limberis MP, Gao GP, Van Vliet K, et al. The AAV9 receptor and its modification to improve *in vivo* lung gene transfer in mice. *J Clin Invest.* 2011; 121: 2427- 2435.
25. Arany Z, Foo S, Ma Y, Ruas J, Bommi-Reddy A, Giron G, et al. Hif-independent regulation of vegf and angiogenesis by the transcriptional coactivator pgc-1alpha. *Nature.* 2008; 451: 1008-1012.
26. Yan Z, Okutsu M, Akhtar Y, Lira V. Regulation of exercise-induced fiber type transformation, mitochondrial biogenesis, and angiogenesis in skeletal muscle. *J Appl Physiol.* 2011; 110: 264-274.