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Original Article

Molecular events linking cholesterol to Alzheimer's disease and inclusion body myositis in a rabbit model

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Abstract: Alzheimer's disease (AD) is the most common neurodegenerative disorder, characterized by cognitive impairment and dementia, resulting from progressive synaptic dysfunction, loss and neuronal cell death. Inclusion body myositis (IBM) is a skeletal muscle degenerative disease, displaying progressive proximal and distal muscle weakness, in association with muscle fiber atrophy, degeneration and death. Studies have shown that the late onset version of AD (LOAD) and sporadic IBM (sIBM) in muscle share many pathological features, including the presence of extracellular plaques of β-amyloid peptides and intracellular tangles of hyperphosphorylated tau proteins. High blood cholesterol is suggested to be a risk factor for LOAD. Many neuropathological changes of LOAD can be reproduced by feeding rabbits a 2% enriched cholesterol diet for 12 weeks. The cholesterol fed rabbit model also simultaneously develops sIBM like pathology, which makes it an ideal model to study the molecular mechanisms common to the development of both diseases. In the present study, we determined the changes of gene expression in rabbit brain and muscle during the progression of LOAD and sIBM pathology using a custom rabbit nucleotide microarray, followed by qRT-PCR analyses. Out of 869 unique transcripts screened, 47 genes showed differential expression between the control and the cholesterol-treated group during the 12 week period and 19 changed transcripts appeared to be common to LOAD and sIBM. The most notable changes are the upregulation of the hemoglobin gene family and the downregulation of the genes required for mitochondrial oxidative phosphorylation in both brain and muscle tissues throughout the time course. The significant overlap on the changes of gene expression in the brain and muscle of rabbits fed with cholesterol-enriched diet supports the notion that LOAD and sIBM may share a common etiology.

Keywords: Alzheimer's disease, rabbit AD model, sIBM, nucleotide microarray, gene expression, hemoglobin, mitochondrial oxidative phosphorylation

Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disorder that displays clinical symptoms of cognitive impairment and dementia. Accumulation of extracellular β-amyloid (Aβ) plaques, composed of Aβ peptide, and intracellular neurofibrillary tangles, containing aggregated and hyperphosphorylated tau protein are key hallmarks of the disease [1]. Genetic mutations of three different genes *APP*, *PSEN 1* and *PSEN 2* are associated with early onset familial form of AD (FAD) [2]. The causative factors for the accumulation of Aβ in the sporadic, late onset forms of AD (LOAD) are not known. Only *apolipoprotein E4* (*APOE4*) has been established unequivocally as a susceptibility gene for LOAD [3]. Two large-scale Genome-Wide Association Studies in large patient cohorts have identified clusterin, also known as apolipoprotein J, as being independently associated with LOAD [4]. Both ApoE and ApoJ are involved in lipid metabolism/homeostasis as components of HDL [5, 6]. Epidemiological and laboratory studies have linked hypercholesterolemia to increased Aβ production and AD pathogenesis [7-10]. However, molecular mechanisms by which cholesterol causes Aβ accumulation and contributes to AD pathogenesis still remain elusive.

Sporadic inclusion body myositis (sIBM) is a degenerative muscle disease that manifests slowly progressive proximal and distal muscle

weakness, in association with muscle fiber atrophy, degeneration and death [11]. Pathological features of sIBM include the presence of dysmorphic muscle fibers and vacuoles, increased numbers of inflammatory cells, accumulations of Aβ and hyperphosphorylated tau, in the form of paired helical filaments [12]. The exact pathogenesis of this disease is unknown and no effective treatment has yet been found.

Pathological aspects of sIBM show intriguing overlap with those displayed in AD. The respective molecular events leading to their similar pathological features are not well understood. Cell aging, aberrant protein processing, folding and turnover, excessive oxidative stress and insufficient antioxidant defenses, mitochondrial abnormalities, altered cholesterol synthesis and transport and chronic inflammation have been proposed to contribute to AD and sIBM [13-17]. Since both AD and sIBM are found in advanced age and the diagnostic linkage is often broken once either of the diagnosis has been established, it is not known whether all cases of sIBM eventually develop dementia. Two recently reported cases of patients with sIBM who later developed AD clearly support the notion that AD and sIBM share common etiology of [18, 19].

Studies by several groups, including ours, demonstrate that the cholesterol fed rabbit LOAD model develops full-blown AD pathology. This includes cortical Aβ deposits and tangles, and up to twelve other pathological markers also seen in human AD brains [20-25]. The rabbit model also simultaneously exhibits human-like sIBM pathological features [26]. In the present study, we took advantage of this dual rabbit model, aimed to identify genes that changed their expression levels during the progression of LOAD and sIBM. The gene expression profiling was carried out using custom rabbit oligonucleotide microarrays and confirmed with subsequent quantitative RT-PCR (qRT-PCR) analysis on brain and muscle RNAs extracted from time-course control and cholesterol fed rabbits. Our objective was to identify common molecular changes that can provide better understanding of disease development and the causes and effects of various pathologic aspects related to LOAD and sIBM. Array data analysis revealed that 47 genes showed differential expression between the control and the cholesterol-treated group during the 12 week period and 19 changed transcripts appeared to be common to LOAD and sIBM.

Materials and methods

Experimental animals and laboratory procedures

Male New Zealand white rabbits were used in this study (2 year old, weighing 3-4 kg). Animals were randomly assigned to two different groups. Group 1 was fed normal chow. Group 2 was fed chow supplemented with 2% cholesterol (Harlan Teklad Global Diets, Madison, WI). Diets were kept frozen at -10°C to reduce the risk of oxidation. The animals were allowed water filtered through activated carbon filters. One control and two cholesterol-treated rabbits were euthanized with ear intravenous injection of euthasol at 2, 4, 6, 8, 10 or 12 weeks of diet regiments. At necropsy, animals were perfused with Dulbecco's phosphate-buffered saline at 37°C and the brains were promptly removed. Frontal Cortex and muscle from forelimb (triceps) tissue were then dissected for further analysis. All animal procedures were carried out in accordance with the U.S. Public Health Service Policy on the Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the University of North Dakota.

Microarray production

The custom rabbit probe set contained 893 5' amine labeled 70 mer oligo nucleotides, representing 869 unique transcripts. The rabbit probe set was designed from a collection of human AD, blood brain barrier/inflammation related genes from literature, genes obtained by subtractive hybridization using RNAs from AD and control human brains [14] and genes obtained from our in house preliminary proteomics analysis comparing the brain and muscle tissues of control rabbits with those of 4 week, 8 week and 12 week cholesterol fed rabbits (unpublished results). The probe set and two negative control oligos (synthesized by Eurofins MWG Operon, AL, USA) were printed onto epoxysilane coated Nexterion® slide E (Schott North America, KY, USA) using a NanoPlotter (NP2.1, GeSiM, Germany). The probes were resuspended in 2X Nexterion spotting buffer, spotted at 8 ± 2°C and a relative humid-

Table 1. Experimental design for two-color microarray experiment

Control vs. Treated group 1		Control vs. Treated group 2	
Cy3	Cy5	Cy3	Cy5
T2	C2	T12	C6
T2	C4	T12	C2
C2	T4	C2	T2
T4	C4	T10	C2
T4	C6	T10	C4
C4	T6	C4	T12
T6	C6	T8	C4
T6	C8	T8	C6
C6	T8	C6	T10
T8	C8	T6	C6
T8	C10	T6	C8
C8	T10	C8	T8
T10	C10	T4	C8
T10	C12	T4	C10
C10	T12	C10	T6
T12	C12	T2	C10
T12	C2	T2	C12
C12	T2	C12	T4

C corresponds to the control group. T represents the treated groups. The number next to C and T corresponds to the weeks of cholesterol treatment.

ity of 50%. After printing, the slides were left in the printer for 2 hours. The slides were then incubated at 42°C and 50% humidity for overnight before storage in a desiccator until use. The spotted probe set on the slide was verified by hybridizing a sample slide with Cy3-9 mer (QIAGEN, Valencia, CA). Missing or low density spots, due to low probe concentration were flagged.

DNA microarray analysis

Total RNA was isolated from cerebral cortex and forelimb muscle samples using the *mir*Vana™ miRNA Isolation Kit and the large and small RNAs were separated according to the manufacturer's instruction (Ambion, TX, USA). The quality of large and small RNAs was verified by electrophoresis with 15% acrylamide gel. Extracted large RNAs (1 μg per sample) from control or cholesterol treated rabbit samples were labeled with Cy3 or Cy5 fluorescent dye using 3DNA Array 900 kit (Genisphere, PA, USA). The Cy3 and Cy5 labeled samples were mixed pair-wise and hybridized to the arrays in a manner resulting in an equal number of data set for each dye and each sample. A total of 36 different microarrays were performed for each experiment (**Table 1**). Probe hybridization was performed using a SlideBooster SB400 hybridization station at 42°C. The hybridization and wash procedures were performed according to the manufacturer's instructions (Genisphere, PA, USA). The slides were then scanned for the fluorescence intensities using a GenePix 4200A Scanner.

Data analysis and normalization

Data extraction and image analysis were performed using GenePix Pro software (Axon Instruments, Molecular Devices Corp. USA). The resulting data was processed by applying a local background subtraction. Replicate spots from the same array, from the control groups and from the two different treated groups of the same week of cholesterol treatments were averaged. Transcripts showing significant differences between cholesterol treated and control group were collected after normalization with the Cyclic Loess algorithm using the Limma package in Bioconductor. The default parameters were used during this normalization. A p value of less than 0.05, a minimum intensity threshold requirement of 8 (from a 16 pixels scanning) and 1.35 fold of changes in ratio were used to generate a gene list.

Quantitative RT-PCR

The RT-PCR experiments were performed on RNA samples at 4 week-intervals during the time course, consisting of the existing two samples for each time point used from the microarray experiments and three additional control and cholesterol treated samples for each time point, except the 8 week cholesterol treatment (with 2 left, one 8 week animal died during treatment). First strand cDNA synthesis, and qRT-PCR analysis were performed as described previously [14]. Fluorescent products were detected using an Applied Biosystems 7500 fast real-time PCR system (Applied Biosystems Inc. Foster City, CA). The q-RT-PCR experiments were performed in duplicate for each sample. Housekeeping genes RPL30 and RPL37 were used to normalize the qRT-PCR results. Only significant differences ($\rho < 0.05$; t-test on the qRT-PCR experiments) between treated and control samples are reported as differentially expressed genes.

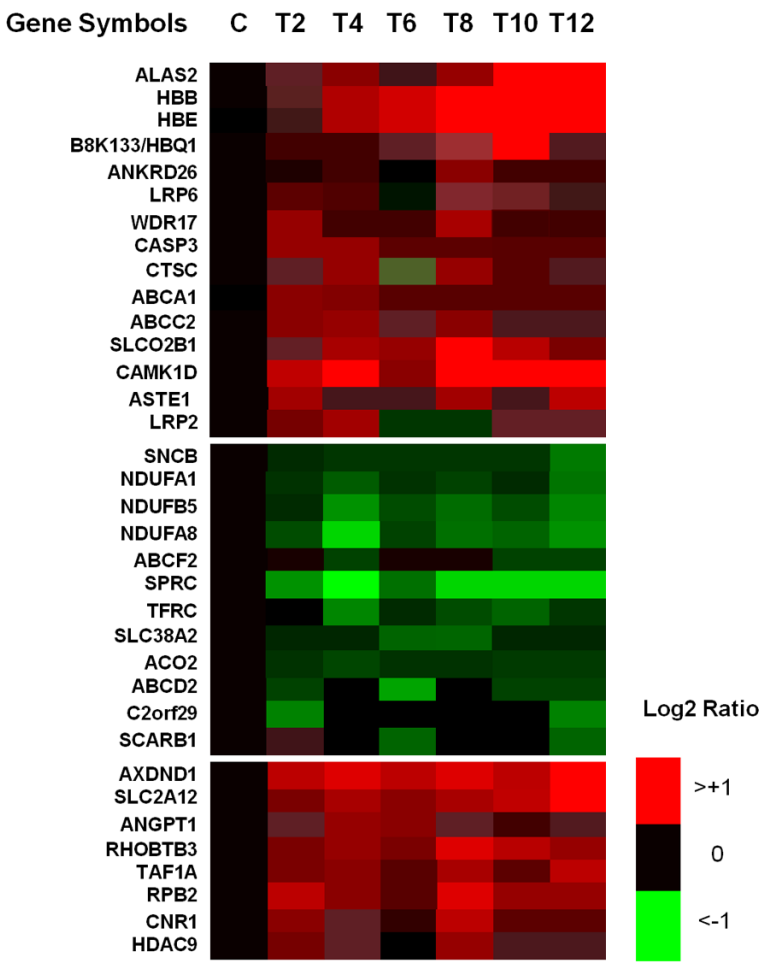

Figure 1. Heat map of expression profile of genes in rabbit brain during the progression of AD and sIBM. After feeding rabbits with diets supplemented with 2% cholesterol over a period of 12 weeks, large total RNAs were extracted from their cortex and studied by microarray analysis. Up regulated genes are shown in red and down regulated genes are shown in green. The different intensities of the colors are proportional to the $\log_2$ of the ratio of the genes between the treated and control samples. The column labeled C represents the average of all the control samples. The columns labeled T represent the average of the two treated samples. The number next to T corresponds to the weeks of treatment. A total of 35 genes showed changes in expression between the control and the treated groups. The tope panel contains genes that are up regulated in both brain and muscle samples, the middle panel contains down regulated genes in brain samples and the bottom panel contains genes that are only up regulated only in brain samples. >1 is more than 2 fold up, < -1 is more than 2 fold down relative to control. The control is 0 in all cases.

Results

Genes changed their expression profile in rabbit brain during the time course of Alzheimer's disease progression defined by microarray analysis

In order to identify differentially expressed genes during the progression of cholesterol induced AD, we examined the expression profiles of 869 candidate rabbit genes at two-week intervals during the course (spanning 12 weeks) of the cholesterol feeding. Data analysis revealed that 35 genes showed changes in expression between the control and the treated groups. These results are represented as a heat map of the $\log_2$ ratio of the cholesterol-fed versus the control group (**Figure 1**). Twenty three transcripts showed trend of upregulation in two or more time points and most of them started to increase early in the treatment; none of them represents genes that are only up regulated at the end point of the treatment, when the AD pathology and neurodegeneration had become apparent (**Figure 1** top and bottom panel) [20-22, 24, 25]. Among the up regulated genes, 3 hemoglobin genes (HBB, HBE and HBQ1) and 5'-Aminolevulinate Synthase 2 (ALAS2), an enzyme that catalyzes the first step in the heme biosynthetic pathway, were notably up regulated throughout the cholesterol treatment. Several transporters (ABCA1, MRP2, SLCO2B1, and SLA2A12) and two low density lipoprotein receptor-related proteins were also in the up regulated category.

Twelve transcripts were found to be down regulated in treated rabbit brain samples. Several genes required for mitochondrial oxidative phosphorylation (NDUFA1, UDUFB5 and NDUFA8) were decreased in cholesterol fed rabbit brains. The β-synuclein gene (SNCB) that encodes a neuroprotective peptide was found to be down regulated in the cholesterol-fed rabbit brains. Transferrin receptor (TFRC) was down regulated in the middle of the time

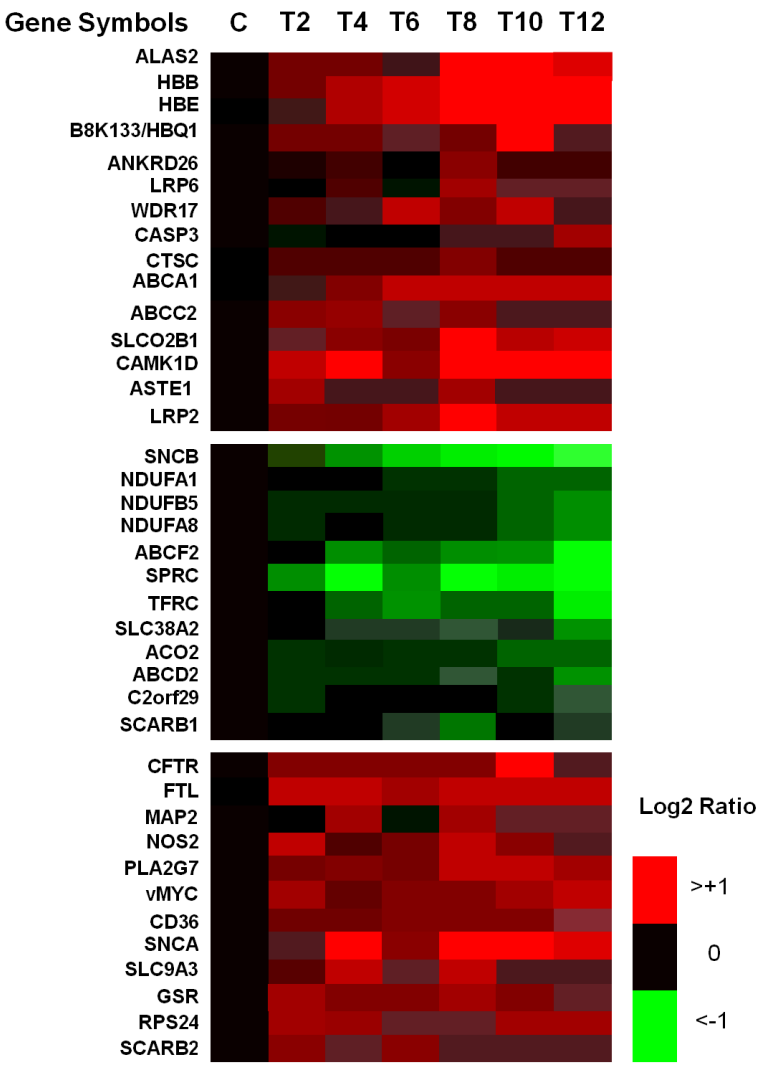

Figure 2. Heat map of expression profile of genes in rabbit muscle during the progression of AD and sIBM. After feeding rabbits with diets supplemented with 2% cholesterol over a period of 12 weeks, large total RNAs were extracted from their forelimb muscle and studied by microarray analysis. Up regulated genes are shown in red and down regulated genes are shown in green. The different intensities of the colors are proportional to the $\log_2$ of the ratio of the genes between the treated and control samples. The column labeled C represents the average of all the control samples. The columns labeled T represent the average of the two treated samples. The number next to T corresponds to the weeks of treatment. A total of 45 genes showed changes in expression between the control and the treated groups. The tope panel contains genes that are up regulated in both brain and muscle samples, the middle panel contains down regulated genes in muscle samples and the bottom panel contains genes that are up regulated only in muscle samples. >1 is more than 2 fold up, < -1 is more than 2 fold down relative to control. The control is 0 in all cases.

course. Surprisingly, secreted protein, acidic cysteine-rich (Osteonectin, SPARC) was the most down regulated gene throughout the cholesterol feeding regimen (**Figure 1** middle panel).

Genes changed their expression profile in the muscle samples in parallel with the time course of Alzheimer's disease progression defined by microarray analysis

We have previously established that rabbits fed with cholesterol-enriched diet exhibit pathological features of sIBM in skeletal muscle and some of those features are common pathological hallmarks of AD [26]. In this study, we were curious to find out whether there exist overlapping gene expression profiles in the diseased rabbit brain and muscle. Microarray data analysis revealed 39 genes changed their expression levels. Of the 27 up regulated genes in the muscle samples 15 showed good overlap with the brain samples, including hemoglobin related genes, low density lipoprotein receptor-related proteins and transporters (**Figure 2** top panel). Twelve genes up regulated in the muscle samples were not changed in the brain samples (**Figure 2** bottom panel). Five of these muscle unique genes showed strong upregulation by microarray analysis, including ferritin light chain (FTL), inducible nitric oxide synthase (NOS2), phospholipase A2, group VII (PLA2G7), C-Myc proto-oncogene (v-MYC) and synuclein alpha (SNCA). Similar to the brain samples, the up regulated genes were detected almost throughout the cholesterol treatment. Most notable changes were found around 8 weeks.

All down regulated genes in the muscle discovered by microarray analysis overlap with those of brain samples (**Figure 2** middle panel). SNCB and SPARC are the most down regulated genes. In the muscle tissue, most notable decreases

Table 2. Summary of qRT-PCR analysis of the up regulated genes in brain tissue

Gene Symbol	4 Week	8 Week	12 week
ALAS2	++	+++	++++
HBB	++++	++++	++++
HBE	++++	++++	++++
HBQ1		+	
ANKRD26			
LRP6			
WDR17		+	
CASP3			
CTSC	+	+++	
ABCA1	+	+	+
ABCC2	+	+	
SLCO2B1	+	++	+
CAMK1D	++++	++++	+++
ASTE1	+		+
LRP2	+	-	+
AXDND1	+++		+
SLC2A12	+	+++	+++
ANGPT1	+	+	+
RHOBTB3	+	++	+
TAF1A	+++	+	+++
RPB2			
CNR1			
HDAC9			

Total large RNAs were extracted from the cortex of control and cholesterol treated rabbits. The qRT-PCR experiments were performed on RNA samples at 4 week-intervals during the time course (n=4 for 8week cholesterol treated and n=5 for all other time points). Each qRT-PCR experiment was performed in duplicate. The PCR cycle numbers of each sample group were normalized against the average cycles of the housekeeping genes RPL30 and RPL37 to obtain ΔCts and then compared with the control group to obtain the ΔΔCts; where, ΔΔCt range from 0.33 to 0.58 was recorded as "+", range from 0.6 to 0.81was recorded as"++", range from 0.82 to 1 was recorded as "+++" and over 1 was recorded as "++++". Only significant differences ($\rho < 0.05$; t-test on the qRT-PCR experiments) between treated and control samples are reported as differentially expressed genes.

Table 3. Summary of qRT-PCR analysis of the up regulated genes in muscle tissue

Gene Symbol	4 Week	8 Week	12 week
ALAS2		++++	++++
HBB	++++	++++	++++
HBE	+++	++++	++++
HBQ1		++	
ANKRD26			
LRP6			
WDR17		++	
CASP3			
CTSC	+	++	+
ABCA1	++++	++++	++++
ABCC2	+	+	
SLCO2B1		++++	++
CAMK1D	++	++++	++
ASTE1		+	
LRP2		++++	++
CFTR	++	+	
FTL	++	++++	++++
MAP2	+	+	
NOS2	++	++++	++
PLA2G7	+	++++	++++
v-MYC	++++	++	++++
CD36	+		++
SNCA	++++	++++	++++
SL9A3	+		
GSR			
RPS24			
SCARB2			

Total large RNAs were extracted from the forelimb muscle of control and cholesterol treated rabbits. The qRT-PCR experiments were performed on RNA samples at 4 week-intervals during the time course (n=4 for 8week cholesterol treated and n=5 for all other time points). Each qRT-PCR experiment was performed in duplicate. The PCR cycle numbers of each sample group were normalized against the average cycles of the housekeeping genes RPL30 and RPL37 to obtain ΔCts and then compared with the control group to obtain the ΔΔCts; where, ΔΔCt range from 0.33 to 0.58 was recorded as "+", range from 0.6 to 0.81was recorded as"++", range from 0.82 to 1 was recorded as "+++" and over 1 was recorded as "++++". Only significant differences ($\rho < 0.05$; t-test on the qRT-PCR experiments) between treated and control samples are reported as differentially expressed genes.

appeared to be in the 12 week cholesterol treated samples.

Quantitative RT-PCR validation of identified by microarray analysis

The expression levels of all 45 changed transcripts, putatively identified by microarray experiments, were verified on both brain and muscle tissues by qRT-PCR analysis using 5 animals (4 animals at 8 weeks) at 4 week-intervals during the time course. Three out 15 up regulated genes shared by brain and muscle tissues were not confirmed by qRT-PCR analysis. They were ANKRD26, LRP6 and CASP3. Other genes displayed in the top panels of both

Table 4. Summary of qRT-PCR analysis of the down regulated genes in brain tissue

Gene Symbol	4 Week	8 Week	12 week
SNCB		--	
NDUFA1	-		-
NDUFB5	-		-
NDUFA8	--	-	--
ABCF2			-
SPARC	----	--	---
TFRC	-		
SLC38A2		-	
ACO2			
ABCD2			
C2orf29			
SCARB1			

Total large RNAs were extracted from the cortex of control and cholesterol treated rabbits. The qRT-PCR experiments were performed on RNA samples at 4 week-intervals during the time course (n=4 for 8week cholesterol treated and n=5 for all other time points). Each qRT-PCR experiment was performed in duplicate. The PCR cycle numbers of each sample group were normalized against the average cycles of the housekeeping genes RPL30 and RPL37 to obtain ΔCts and then compared with the control group to obtain the ΔΔCts; where, ΔΔCt range from -0.33 to -0.58 was recorded as "-", range from -0.6 to -0.81was recorded as"--", range from- 0.82 to -1 was recorded as "---" and below -1 was recorded as "----". Only significant differences ($\rho < 0.05$; t-test on the qRT-PCR experiments) between treated and control samples are reported as differentially expressed genes.

Table 5. Summary of qRT-PCR analysis of the down regulated genes in muscle tissue

Gene Symbol	4 Week	8 Week	12 week
SNCB	----	----	----
NDUFA1			-
NDUFB5			--
NDUFA8			-
ABCF2	-	-	---
SPARC	----	----	----
TFRC	-	--	----
SLC38A2			--
ACO2			--
ABCD2			--
C2orf29			--
SCARB1			

Total large RNAs were extracted from the forelimb muscle of control and cholesterol treated rabbits. The qRT-PCR experiments were performed on RNA samples at 4 week-intervals during the time course (n=4 for 8week cholesterol treated and n=5 for all other time points). Each qRT-PCR experiment was performed in duplicate. The PCR cycle numbers of each sample group were normalized against the average cycles of the housekeeping genes RPL30 and RPL37 to obtain ΔCts and then compared with the control group to obtain the ΔΔCts; where, ΔΔCt range from -0.33 to -0.58 was recorded as "-", range from -0.6 to -0.81was recorded as"--", range from -0.82 to -1 was recorded as "---" and below -1 was recorded as "----". Only significant differences ($\rho < 0.05$; t-test on the qRT-PCR experiments) between treated and control samples are reported as differentially expressed genes.

heat maps were all confirmed to be genuinely up regulated (**Figures 1** and **2**). The levels of up regulation for genes in rabbit brain and muscles after cholesterol feeding as confirmed by qRT-PCR are listed in **Tables 2** and **3**, respectively. For genes only up regulated in cholesterol treated brain tissue (**Figure 1** bottom panel), RPB2, CNR1 and HDAC9 were not confirmed by qRT-PCR (**Table 2**). Of the 12 genes uniquely up regulated in cholesterol treated muscle tissue (**Figure 2** bottom panel), GSR, RPS24 and SCARB2 were not confirmed by qRT-PCR (**Table 3**). In the group of genes showed expression pattern of downregulation in both brain and muscle tissues by microarray analysis, SLC38A2, ACO2, ABCD2, C2orf29 and SCARB1 were found to be not significantly down regulated in the brain by qRT-PCR (**Table 4**). Only one gene, SCARB1 was not confirmed to be genuinely down regulated in cholesterol treated muscle tissue (**Table 5**).

Discussions

The cholesterol-fed rabbit has been accepted as a model for AD, because it models the human sporadic late-onset Alzheimer's disease in many pathological aspects [20-25]. This model has also been extended to the studies of sIBM, a disease that is pathologically linked to some features of AD [26]. Our recently published work, as well as ongoing research focuses on the characterization of this model at the molecular level, including microRNA profiling and metabolomics [27, 28]. The present study used a rabbit-specific custom oligo nucleotide DNA microarray, developed in house, to profile the time-course of cholesterol fed rabbit brain and muscle tissues in order to identify critical molecular signatures that are common to LOAD and sIBM. After qRT-PCR verification, we found 12 up regulated and 7 down regulated genes were commonly altered by cholesterol-enriched

diet in both brain and muscle tissues (**Tables 2**-**5**). In addition, several genes, such as ferritin light chain (FTL), microtubule-associated protein 2 (MAP2), inducible nitric oxide synthase (NOS2) and CD36 antigen (CD36), known to be up regulated in human AD brains [14, 29-31], were significantly up regulated in cholesterol treated muscle tissues. Similarly, aconitase 2, mitochondrial (ACO2) reported to be down regulated in the lymphocytes of AD and MCI patients was also down regulated in cholesterol treated muscle tissues [32]. It is possible that these genes could also be altered in the cholesterol-fed rabbit brains, but did not pass our statistical cut off due to animal variations. Taken together, our results suggest that there is a significant overlap on the changes of gene expression in the brain and muscle of rabbits fed with cholesterol-enriched diet, supporting the notion that sporadic Alzheimer's disease and inclusion body myositis may share a common etiology [13, 19]. Although we detected stronger changes of several genes in muscle than brain at certain time points, it is not possible at present to determine temporal sequences of events in the brain and muscle.

One of the most significant changes in gene expression in this rabbit model is the upregulation of the hemoglobin gene family, including the aminolevulinate, delta-, synthase 2, which catalyzes the first step in the home biosynthetic pathway. It has long been reported that hemoglobin (Hb) β chain transcript is up regulated in postmortem AD brains and hemoglobin binds to Aβ and co-localizes in amyloid plaques and cerebral amyloid angiopathy in AD brains [33, 34]. It was later demonstrated that the interaction between Hb and Aβ is heme dependent [35]. Hb is the most abundant oxygen carrying protein in human body. Several studies demonstrated that Hb and ALAS2 gene expression is regulated by hypoxia [36-38]. Hypoperfusion associated cerebral hypoxia is often observed in aged and AD brains [39]. Upregulation of Hb could be an intrinsic mechanism to facilitate cellular oxygenation. Aged and AD brains are frequently associated with profound changes in brain vascular structure and function [39]. At the site of capillary hemorrhage, hemoglobin released into the neuropil binds Aβ and promotes oligomerization, which provides an explanation of why plaques form around capillaries as the capillary bed becomes fragile with age and why heme is found in every plaque in sporadic and familial dementia patients [40]. In this cholesterol induced rabbit model, we observed dramatic up regulation of the Hb family genes almost immediately after the cholesterol treatment (2-4 weeks). This outcome cannot be attributed to ageing associated hypoperfusion. However, it might solely be caused by cholesterol overload, where increased plasma cholesterol impairs the red blood cell membrane, which in turn, reduces oxygen transport [41]. This condition in rabbit brain and muscle mimics the hypoxia environments in aged and AD brain; therefore induces an upregulation of Hb gene family. Hb/heme and iron overload can not only induce cytotoxicity, but also contribute to Aβ deposition at the vascular injury site in the later stage of cholesterol treatment.

Another significant change affecting a gene family is the down regulation of the genes required for mitochondrial oxidative phosphorylation (NDUFA1, NDUFB5 and NDUFA8). It has been reported that high fat diet increases the flux of fatty acids through skeletal muscle for oxidation, which might be interpreted as a signal of fasting/starvation by the muscle cell itself. This condition is clearly associated with reduction in the expression of genes involved in oxidative capacity, including, NDUFB5 [42]. In rabbits fed with cholesterol-enriched diet, we found genes involved in mitochondrial oxidative phosphorylation down regulated in both muscle and brain tissues, suggesting that defects in mitochondrial oxidative phosphorylation induced by high dietary fat are not limited to muscle cells. If this condition persists for a prolonged period, it may become a factor contributing to neuronal and muscle degeneration leading to LOAD and sIBM.

One dramatic change in gene expression in both brain and muscle is the downregulation of SPARC transcript (**Tables 4** and **5**). Kong and colleagues [43] reported the changes of SPARC transcript levels in AD brains using DNA microarray data from public data bases. However, the results are somewhat contradictory, since it was shown to be up regulated by independent component analysis (ICA), but down regulated by principal component analysis (PCA). In the present study, we found SPARC transcript to be dramatically decreased throughout the choles-

terol treatment. Since SPARC is a secreted peptide, if it is detectable in body fluids and confirmed to be changed on protein levels, it could be further developed into an early biomarker for LOAD and sIBM.

In agreement with previous findings in human AD brain, the neuroprotective SNCB is down regulated in both brain and muscle of this rabbit model [44]. Several reports have also shown that SNCA is down regulated in human AD brains [14, 45, 46]. The change of SNCA in the brain of this rabbit LOAD model is not significant by both microarray and q-RT-PCR analyses. However, we were surprised to find the SNCA transcript significantly up regulated in the muscle of the cholesterol treated rabbits. SNCA is expressed in both neurons and non-neuronal cells, such as muscles, spleen, and endothelial cells [47]. Although the brain is suggested to be the major source of SNCA production, red blood cells also contain SNCA [48]. The SNCA gene has also been shown to be expressed in both neuronal and hematopoietic cells. These latter results strongly suggest that SNCA transcription can be activated both centrally and peripherally [49]. Askanas and colleagues [50] found abnormal accumulation of SNCA in human Aβ-positive vacuolated muscle fiber. It is not clear at present whether the upregulation in mRNA levels contribute to its pathological deposition in protein level, but this upregulation appears to be unique to muscle tissue in this model.

In conclusions, we have identified a number of genes differentially expressed in the cholesterol-fed rabbit brain and muscle tissues that are common to human AD, suggesting that there exist similar molecular mechanisms between this rabbit LOAD model and human AD development. More importantly, we found 19 changed genes overlap between brains and muscle tissues in this dual rabbit model. Many of these shared changes occurred early in the time course, leading to a speculation that these genes participate in disease development. Our results also confirm the notion that LOAD and sIBM share molecular events leading to their disease pathology. Since the accessibility of muscle is much easier than brain, in terms tissue biopsy and drug delivery, the advance of sIBM diagnosis, prevention, and treatment could facilitate those for LOAD.

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Disclosure of conflict of interest

None.

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