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Article

Exosome TDP-43 profile in canine model of ALS: A preliminary study in developing surrogate biomarker

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Abstract: Blood-based biomarkers are considered non-invasive and have the potential to be cost effective. Validated blood-based biomarker application in people with amyotrophic lateral sclerosis (ALS) would derive numerous benefits. Canine degenerative myelopathy (DM) is a animal disease model to study the biology of human ALS. Serum derived exosomes are potential carriers for cell-specific cargoes of proteins, lipids, and genetic materials making them ideal biomarkers for a variety of diseases and biological processes. This study examined the exosomal trans active response DNA binding protein 43 kDa (TDP-43). The TDP-43 pattern as a surrogate biomarker that reflects biochemical changes in central nervous system. Exomes were isolated from canine serum using commercial exosome isolation reagents. TDP-43 and SOD1 profiles in spinal cord homogenate lysate and that of serum-derived exosomes were found elevated in dogs with DM. The authors concluded that levels of spinal cord TDP-43 and serum-derived exosomes were similar in TDP-43 profiling Further investigations are warranted to establish the disease sensitivity and specificity of the exosomal TDP-43 profile as a blood-based biomarker in canine DM.

Keywords: neurodegeneration; serum; spinal cord; dog, Degenerative myelopathy, amyotrophic lateral sclerosis

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1. Introduction

The aberrant protein aggregation in motor neurons is the hallmark of amyotrophic lateral sclerosis (ALS). ALS is a progressive disease that directly affects motor neurons, leading to loss of muscle function. About 5-10% of the inherited forms of ALS are linked to a mutation in the SOD1 gene *(SOD1).* The misfolded mutant [Cu/Zn] superoxide dismutase (SOD1) protein is believed to contribute to development of ALS, yet the role of misfolded non-mutant SOD1 in the disease progress is unclear. Recently, chemically modified aberrant Transactive Response DNA Binding Protein 43 (TDP-43) species were found to represent a major accumulating protein in neuronal cytoplasmic inclusions [1, 2] and in exosomes [3, 4] in Frontotemporal Lobar Degeneration (FTLD), and in ALS patients [5]. Exosomes are nano-size membranous vesicles that contains several macromolecules including aberrant pathological proteins. The size and membranous structure of exosomes allow them to pass through the blood brain barrier. These features make exosomes a potential platform in which targeted biomolecules can be analyzed. The bio-molecular link and clinical similarities between human ALS and canine DM suggested that human ALS hallmark signature proteins (i.e., SOD1 and TDP-43) have the same or similar profile in canine DM. Therefore, canine DM animal model may be employed to further biomarker validity studies such as reproducibility, specificity, sensitivity, and robustness.

ALS is an incurable and fatal disease with a patients survival rate of 3-5 years once diagnosed **[6]**. In most patients, the symptoms begin in the lower limbs. Patients often complain of tripping, stumbling, foot drop, or a "slapping" gait. Upper limb involvement includes decreased dexterity in the fingers, cramping, stiffness, weakness and wrist drop. Early diagnosis of ALS is very difficult, which can delay time to treatment **[7]**. Establishing a blood-based biomarker(s) will help clinicians with early diagnosis and initiating timely treatment. Timely pharmaceutical intervention may improve the quality of life and lifespan for patients.

Animal models for ALS have used rodents with mutated SOD1 to study the pathology of the disease [8]. The rodent model is of limited use as disease progression is very short (3-6 months), and is based on overexpression of the human gene for mutant SOD1. In addition, peripheral biomarker studies are difficult to perform in a longitudinal course due to limited access to serial samples of serum/exosomes in mice due to the small volume of blood. Canine degenerative myelopathy (DM) is a late adult neurodegenerative disease accompanied by (*SOD1)* mutations (*SOD1:c.118A,SOD1:c52T*), and protein aggregation [9, 10]. Early clinical signs are general proprioceptive ataxia, and spastic upper motor neuron paresis in the pelvic limbs with progression to flaccid tetraplegia and dysphagia [11-13]. DM and ALS are therefore characterized by progressive multisystem neurodegeneration involving upper and lower motor neurons. Histologic similarities include astrogliosis in the spinal cord, degeneration and loss of myelinated axons of the spinal cord and peripheral nerves and neurogenic muscle atrophy [11, 12].

An abnormal accumulation of TDP-43 has been demonstrated in the cytoplasm and platelets from ALS patients [14] .The post translationally modified derivatives of TDP-43 (i.e., hyper-phosphorylation and ubiquitination)have been established in ALS [15, 16]. Prior research has established that the accumulation of TDP-43 in the cytosol was causal in the loss of function in affected neurons [17, 18]. However, what percent of TDP-43in peripheral blood represents TDP-43 that originates from the central nervous system (CNS)is unknown. Therefore, introducing the exosomal TDP-43 evaluation to encapsulated TDP-43 in exosomes better reflects the aberrant TDP-43 rather than the general population of TDP-43 in blood . This study explored the feasibility of using exosomes to assess potential biomarkers for DM such as TDP-43, pTDP-43, and SOD1.

2. Results

2.1. Dogs with DM spinal cord homogenate show elevated TDP-43 and SOD1 in the thoracic region of the spinal cord

The thoracic region of the spinal cord from companion dogs with DM (n=4) and from age matched non-DM affected control dogs (n=4) were obtained from a tissue archive at the University of Missouri College of Veterinary Medicine. The signalment of dogs used in this study listed in **Table-1** in Supplemental Data. The -80^{0}C frozen spinal cord tissue was homogenized and stored at -80^{0}C , and later subjected to 10% SDS/PAGE followed by western blot analysis. Proteins were immunoprobed with anti-TDP-43 Ab (1/1000 dilution). The intensity of the bands were analyzed by an image analyzer. The fluorescence arbitrary units (a.u.) were normalized to total protein staining. **Figure-1** shows that TDP-43 protein levels are elevated in the DM spinal cord region as compared to that of the control dogs. A t test analysis indicated that differences between two groups were significant ($P< 0.01$). This observation is in agreement of other published data that TDP-43 accumulation has been observed in human spinal cord *[15, 19]*. SOD1-immunoreactive aggregates were observed in ventral horn motor neurons *[20]*. This study confirmed that the spinal cord SOD1 aggregation profile occurred in dogs with DM (**Fig.2**). We considered SOD1 aggregation profile as positive control.

2.2. Exosomal TDP-43 , phosphorylated TDP-43, and SOD1 are elevated in canine with DM

Frozen serum samples from dogs were obtained from a tissue archive at the University Of Missouri College Of Veterinary Medicine. The signalment of dogs used in this study are listed in **Tables2 and 3** in Supplemental Data. Exosomes were isolated from serum by means of vesicle precipitation methods described in the miRCURY exosome isolation kit (Qiagen #76603). Isolated exosome lysates were subjected to SDS/PAGE (4-20% gradient gel) followed by immuno blot analysis. The protein bands were probed with both anti-TDP-43 and anti-pTDP-43 Ab (1/100 dilution). The intensity of the protein bands were analyzed by an image analyzer (LI-COR Odyssey infrared analyzer). The fluorescence arbitrary units (a.u.) were normalized to total protein staining. **Figure-3A** and **3B** showed that both total TDP-43 and its phosphorylated derivative (pTDP-43) were elevated in exosomes of the dogs affected with DM as compared to that of the control dogs. Differences between DM affected and control dogs were found to be significant ($P\leq0.001$).These results suggest that exosomes can be used to assess biomarker proteins such as TDP-43 and its phosphorylated derivatives.

SOD1 proteins in canine serum- derived exosomes exhibit a heterogenic profile (i.e., monomer, trimer and tetramer) (**Fig.4**).Soluble monomers (~25 kDa) were considered in the data analyses and graphing. Dogs affected with DM serum exosomes had elevated SOD1 proteins when compared to control dogs. The difference between the two groups was significant based on an unpaired t-test analysis ($P<0.05$).

2.3. Figures, Tables and Schemes

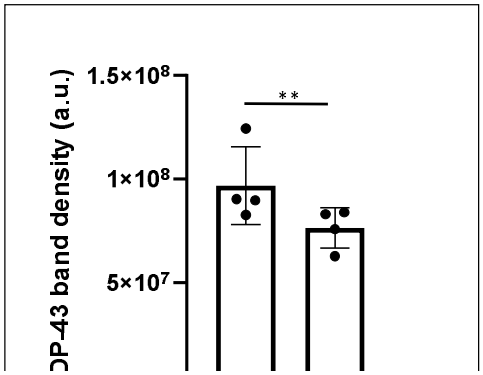

Figure-1. Total TDP-43 levels of thoracic region of spinal cord homogenate. DM affected dogs (n=4, Supplemental Data / Table-1: dog # 1,2,3,4) showed elevated levels of TDP-43 compared to control dogs (n=4, Supplemental Data / Table-1: dog # 5,6,7,8) [One-sample t-test (P<0.01)]. The protein band intensities were normalized to total protein staining. .

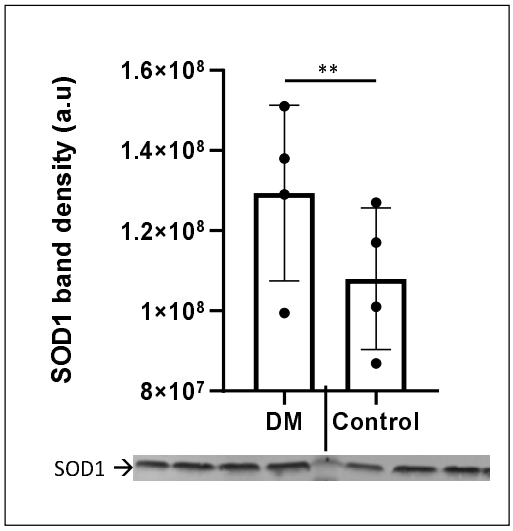

Figure-2. **SOD1 levels of thoracic region of spinal cord homogenate**. DM affected dogs (n=4, Supplemental Data / Table-1: dog # 1,2,3,4) showed elevated levels of SOD1 as compare to control dogs (n=4, Supplemental Data / Table-1: dog # 5,6,7,8) [One-sample t-test (P< 0.01)]. The protein band intensities were normalized to total protein staining.

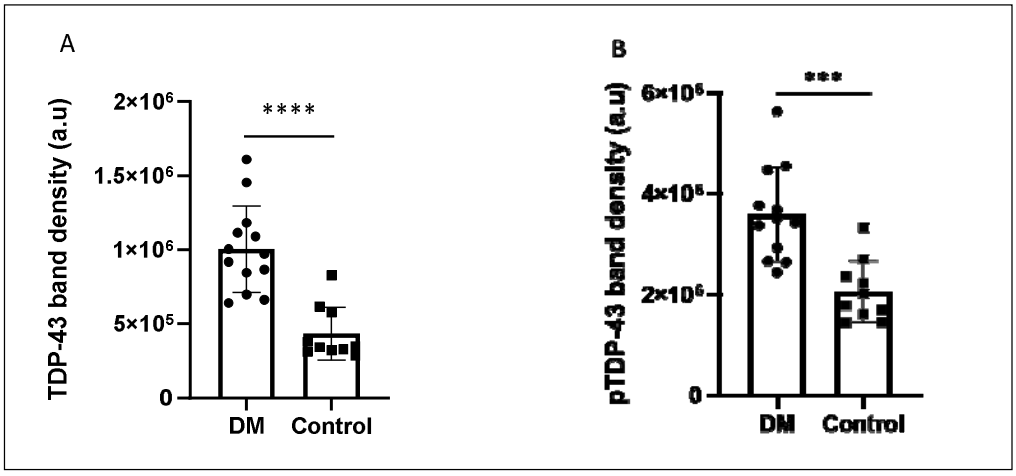

Figure 3. **Serum derived exosomal total TDP-43 and phosphorylated TDP-43 profile in SM affected dogs**. Both total TDP-43 **(A)** and phosphorylated TDP-43 (pTDP-43) **(B)** levels were elevated in serum-derived exosomes from DM affected dogs (n=12; Table-2 Supplemental Data: dog #

1,2,3,4,5,6,7,8,9,10). One-sample t-test analysis revealed statistical significance for total TDP-43 (P<0.0001) and for pTDP-43 (P<0.001) Original immunoblot analysis pertinent to this figure provided in Supplemental Fig.1 and Fig.2. The protein band intensities were normalized to total protein staining.

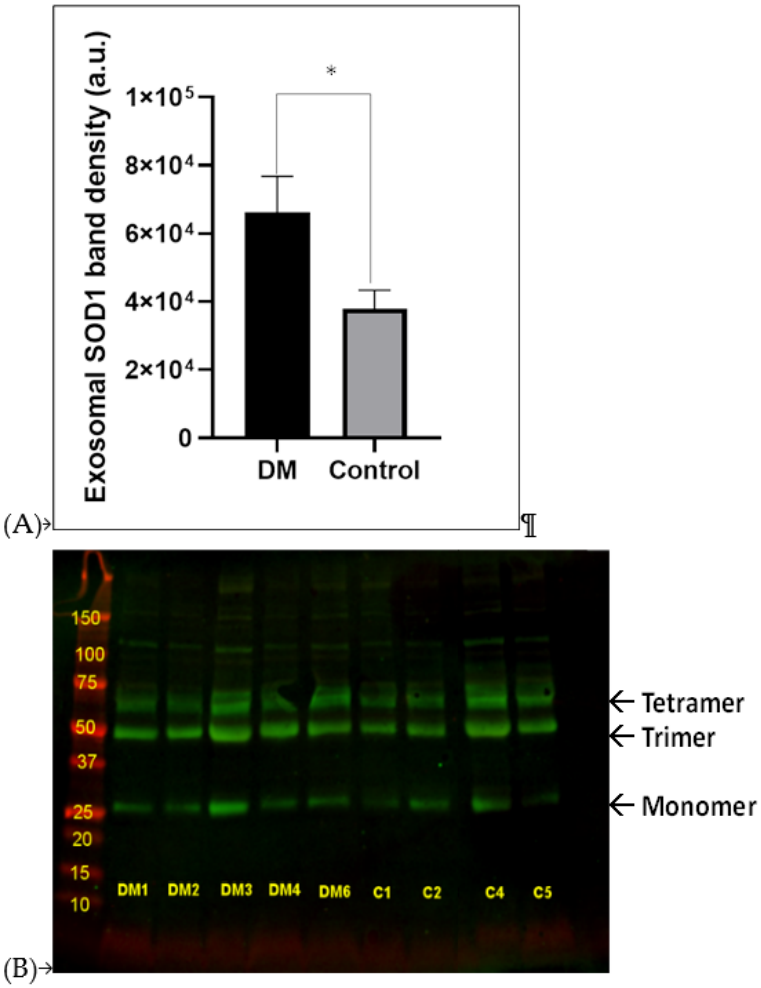

Figure 4. **Serum derived exosomal SOD1 profile in dogs with DM; (A)** Exosomal SOD1 levels appear to be elevated in dogs with DM. The difference between control (n= 3, Table-3 Supplemental Data ; dog #1,2,4,5) and DM (n=4; Table-2 Supplemental Data: dog #1,2,3,4,6) groups did achieve statistically significance (p <0.05). **(B)** Monomer SOD1 proteins bands were analyzed and graphed. The protein band intensities were normalized to total protein staining.

3. Discussion

Complexity of a biosample matrix, and the invasive nature of tissue sampling led investigators to develop peripheral tissue-based biomarkers. Blood-based biomarker initiatives have gained popularity due to easy access for sampling with minimal invasion (i.e., venous puncture), access to several blood cells, and subcellular structures. Once developed and validated, blood-based biomarkers will be utilized for diagnosis, monitoring the prognosis, and monitoring the treatment of ALS [21-23]. In this study, we demonstrated the use of exosomes and its ALS hallmark proteins (i.e., TDP-43, pTDP-43 and SOD1) and compared to that of thoracic spinal cord homogenate. We chose the thoracic region because the disease pathology of canine DM is most severe in this region [24]. This region also reflects more TDP-43 accumulation relevant to pathology of ALS

[25]. Serum exosomes were isolated from the dog cohort consisting of animals with degenerative myelopathy (DM) and age matched non-DM affected.

This study has demonstrated the method for : (i) effectively isolating exosomes from serum;(ii) analyze exosomal TDP-43 and pTDP-43 protein profiles; and (iii)comparing those profiles to spinal cord tissue (thoracic region) homogenate from the different cohort of dogs with DM as part of proof of concept studies. TDP-43 and SOD1 are known as the hallmark proteins for ALS [15, 16, 26]. This study demonstrated thattotal homogenates of spinal cord TDP-43 levels were elevated in dogs with DM (Fig.1). This study also indicated that SOD1 profile served as a positive control and established that both proteins were elevated (Fig.2). The next step will be to demonstrate sensitivity and specificity to establish biomarker reliability.

Exosomes are small stable single-membrane organelles that carry selected proteins, lipids, nucleic acids, and glycoconjugates [27]. Exosome biogenesis is a mechanism of protein quality control [27]. They are emerging as key mediators of communication and waste management among neurons, glial cells and connective tissues during normal and diseases conditions [28]. Although exosomes are being developed as therapeutic agents in multiple disease models, they can also serve as surveillance agents by transporting aberrant proteins like hyper-phosphorylated TDP-43 or cytosolic TDP-43 that could not re-enter into nucleus. Using dog DM established the link between spinal cord and serum/plasma derived exosomes in terms of TDP-43 biochemistry. We have observed increased TDP-43 and SOD1 levels in exosomes derived from dog affected with DM. SOD1analysis was performed to further support spinal cord and serum exosome cross-talk in terms of the content TDP-43 and its derivative. The SOD1 protein profile revealed three SOD1 species (monomer, trimer, and tetramer), although SOD1 resolved under the reducing conditions. Recent observations demonstrated that trimer-SOD1 aggressively promoted cell death [29, 30]Because of their small size, exosomes can easily pass the blood brain barrier to serve as an accessible biomarkers of neuronal dysfunction [31-34]. This preliminary study has established that spinal cord TDP-43 may be transported by exosomes, and that TDP-43 loaded exosomes appear in blood Exosomes derived from serum represent a global exosomes that includes neuronal derived exosomes (Holder). Current research is focusing on the development of a process, to selectively isolate the NDE population from global serum and spinal cord tissue derived exosomes. Their content will be analyzed in terms of TDP-43 and its derivatives.

4. Materials and Methods

4.1. Animals

Previously -80^{0}C frozen spinal cord tissues from thoracic region and serum samples were obtained from Dr. Joan R. Coates at the College of Veterinary Medicine, University of Missouri-Columbia. Tissues were harvested from various breeds with diagnosed DM and age-matched dogs not affected with DM dogs (Table 1 Supplemental Data). All DM affected dogs were homozygous for the SOD1 E40K mutation [13].

4.2. Isolation of exosomes from canine serum

The isolation of exosomes from serum of DM and control dogs was performed using a commercially available particle precipitation method following manufacturer's protocol (miRCURY Exosome Serum/Plasma Kit, Qiagen #76603). The exosome isolation procedure was based on the capture of water molecules, which otherwise would form the hydrate envelope of particles in suspension. Mixing the starting sample with a proprietary precipitation buffer diminished the hydration of the subcellular particles and allowed the precipitation of even particles smaller than 100 nm with a low-speed centrifugation step [36] To remove residual cells, debris, platelets, large micro vesicles etc., the serum samples were first pelleted, and the supernatant was filtered. The samples were incubated with a proprietary precipitation buffer for 1 hour at 4°C. After a 30 minute low

speed centrifugation the exosomes pellets were re-suspended for further characterization. The yield of exosomes yield was verified by immunoprobing assay using an antibody that recognized exosome surface protein, TSG101 (tumor susceptibility 101 protein) (**Supplemental Figure-3**) Exosome suspension was aliquoted and kept in -20^{0}C until use.

4.3. Spinal cord homogenate preparation

50-60 mg thoracic spinal cord tissue (T3 section) was homogenized in an ice-cold buffer (0.32 M sucrose, 0.5 mM $MgSO_4$, 10 mM ε-amino-n-caproic acid, protease inhibitor cocktail .1% v/v, 10 mM HEPES, pH 7.4) in a glass pestle-glass homogenizer (8-10 strokes). The tissue: homogenization buffer ratio was 1(mg tissue):15 (μL buffer). The homogenate was resuspended 8-10 times using a 1 mL syringe with a $26^{6/8}$ gauge needle to shear DNA and liberate nuclear proteins. The homogenate was incubated on ice for 20 minutes followed by centrifugation at 16,000 X g for 20 minutes at 4^{0}C. The supernatant was collected into a clean tube, and contains all soluble proteins, including nuclear proteins. For purpose of the protein assay, 5-10 μL aliquot was saved. The sample was aliquoted into 100 μL volumes into microfuge tubes and stored at -80°C until use.

4.4. Immunoblotting analysis

10 μg of exosomal proteins were loaded onto a 4-20% gradient gel. Electrophoresis was run at 100V for 75-80 min until the dye-front migrated to 0.5 cm from the bottom of the gel. Resolved proteins on the gel were transferred onto a PVDF membrane by electro transfer unit at 60 V for 90 min. The membrane was stained/destained for total protein staining according to manufacturer's protocol (REVERT Total Protein Stain kit, LI-COR, Cat# 926-11016). The membrane was blocked with a blocking agent (SEA BLOCK Blocking, Thermo Scientific, Cat # UH2788881) for one hour at room temperature (RT), followed by overnight incubation with anti- TDP-43 Ab (1:1000 dilution) and anti-SOD1 Ab (1:750 dilution) on an orbital shaker at 4^{0}C. The next day the membrane was incubated with Infrared (IR)-tagged antibody for 1-2 hrs. at RT. The protein bands were visualized in an image analyzer (LI-COR Odyssey Infrared Imager (Model No. 9120). The intensity of protein bands were normalized based on total protein staining signals, and analyzed by Image Studio image analyzing program (V.3.1, Li-COR Biosciences).

Author Contributions: Conceptualization, AA and JRC; methodology, AA, PP, AK,KG, YME, MM and EK.; formal analysis, PP,AK,KG,EK, and AA; writing—original draft preparation, AA; writing—review and editing, AA,MM, and JRC; supervision, AA and EK; All authors have read and agreed to the published version of the manuscript."

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board (or Ethics Committee) of University of Missouri-Columbia (protocol code 8339 and date of 01/15/2015)

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Conflicts of Interest: The authors declare no conflict of interest.

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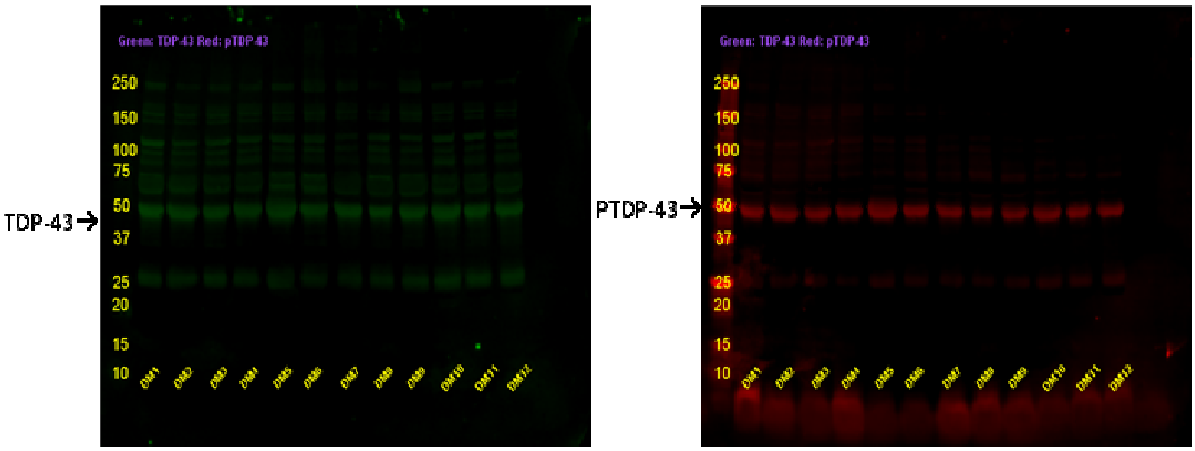
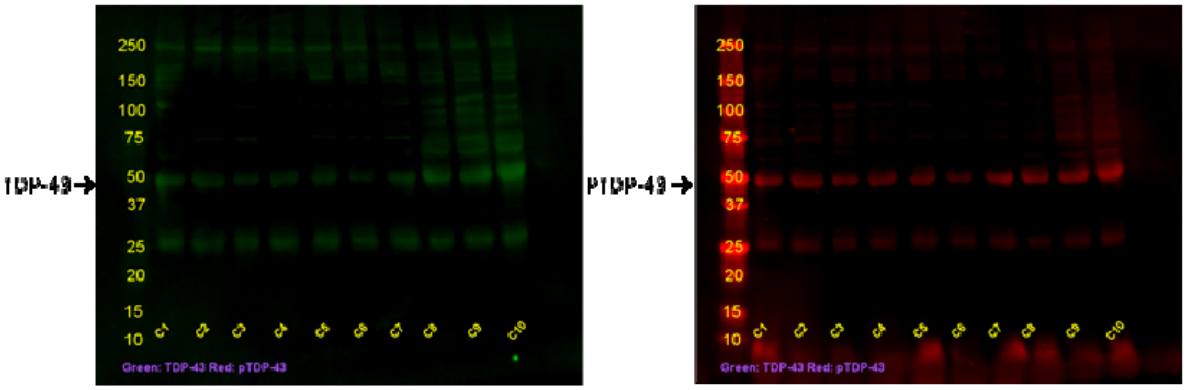
SUPPLEMENTAL FIGURES



Supplemental Figure-1. Serum-derived exosomal TDP-43 and phosphorylated TDP-43 profile in dogs with DM (n=12; Table-2 Supplemental Data : dog # 1,2,3,4,5,6,7,8,9,10,11,12)



Supplemental Figure-2 . Serum derived exosomal TDP-43 and Phosphorylated TDP-43 profile in control dogs (n=10; Table-3 Supplemental Data : dog # 1,2,3,4,5,6,7,8,9,10)

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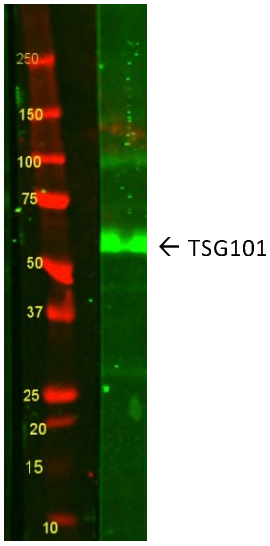

Supplemental Figure-3. Serum/plasma derived exosome isolation and verification by a-TSG101 antibody.