The Establishment of Potential Cerebrospinal Fluid Biomarkers for Canine Degenerative Myelopathy

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Abstract

Canine degenerative myelopathy (DM) is a late onset neurodegenerative disease that primarily affects German Shepherd dog (GSD), though a number of other specific breeds are also affected. The underlying cause of the disorder remains elusive, though recent advances have implicated a mutation of superoxide dismutase 1 (*Sod1*) in the aetiology, also implying DM is a potential orthologue of human amyotrophic lateral sclerosis. The identification of the *Sod*1 mutation raise the index of suspicion for an individual animal, however it is not specifically diagnostic as a proportion of dogs homozygous for the S*od*1 mutation do not develop DM. Therefore, there is a clinical need for the development of specific biomarker(s) for DM to support genetic test.

The aim of this study was to establish potential biomarkers for DM by exploring canine cerebrospinal fluid (CSF). A dual strategy was adopted; 1) Evaluation of potential ALS biomarkers in DM CSF, 2) Identification of novel biomarker(s) in DM CSF. The cases selected in this project had a presumptive diagnosis of DM and were homozygous for *Sod*1 mutation. Preliminary characterisation by Western blot and mass spectrometry identified four protein candidates in DM CSF, comprised of cystatin C, transthyretin (dimeric and monomeric TTR), haptoglobin and clusterin. Since the validity of these putative biomarkers may be influenced by pre-analytical variables that may arise from the clinical environment, we therefore assessed the impact of three potential sample handling practices on these four proteins. The results from these experiments demonstrate that dimeric TTR and clusterin were affected by sample handling conditions. Therefore, an appropriate protocol for CSF sample handling was established.

Western blot analyses indicated that clusterin is the most viable biomarker candidate for DM. Clusterin was significantly elevated in DM CSF when compared to a range of neurological conditions. The second potential candidate for DM biomarker is TTR, which is potentially reduced, an observation similar to those found in ALS CSF. The relationship of these proteins in the pathogenic mechanisms that underpin DM is unclear. However, based on observations on ALS, it is reasonable to speculate that their alterations are associated with a toxic gain of function of the mutant SOD1 protein. The successful characterisation of clusterin and TTR in DM CSF may therefore represent components of a panel of emerging biomarkers that may combine to distinguish DM in the clinic and provide further insights into the disease mechanisms.

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Intan N.F. Shafie, May 2013.

Author's Declaration

I declare that the work presented in this thesis is original, was carried out solely by the author or with due acknowledgement and has not been presented for the award of a degree at any other University.

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Dedication

To my parent and my husband,
Thank you for all the inspiration, unconditional love and support throughout this PhD journey

Abbreviations

%	percentage
<	less than
>	more than
°	degree
°C	degree Celcius
µg	microgram
µl	microlitre
µm	micrometre
µmol	micromole
118G>A	G to A nucleotide transition at 118th position
1-DGE	one-dimensional gel electrophoresis
2-DGE	two-dimensional gel electrophoresis
52A>T	A to T nucleotide transition at 52nd position
7B2	neuroendocrine protein 7B2
A4V	substitution of amino acid alanine to valine at 4th codon
AD	Alzheimer's disease
ALP	alkaline phosphatase
ALS	amyotrophic lateral sclerosis
ALS2	amyotrophic lateral sclerosis 2 (juvenile) gene
ALT	alanine transferase
ANG	angiogenin gene
ANOVA	one-way analysis of variance
APP	acute phase proteins
AST	aspartate transaminase
Ast-HI	astrocytic hyaline inclusion
AVED	ataxia associated vitamin E deficiency
Aβ	amyloid-beta
BCA	bicinchoninic acid
BiP	binding immunoglobulin protein
BMD	Bernese Mountain dog
bp	base pair
BSA	bovine serum albumin

BUN	blood-urea-creatinine
CBC	complete blood count
cDNA	complementary DNA
CDRM	chronic degenerative radiculomyelopathy
CE	capillary-electrophoresis
CFA31	chromosome 31
CHOP	C/Ebp homologous protein
cIVDD	chronic intervertebral disc disease
CKCS	Cavalier King Charles Spaniel
CLU	clusterin
CNS	central nervous system
Complement C3	complement component 3
Con A	concanavalin A
CSF	cerebrospinal fluid
CT	computerised tomography
Cu+	cupric ion
Cys C	cystatin C
D90A	substitution of amino acid aspartic acid to alanine at 90^{th} codon
D90N	substitution of amino acid aspartic acid to asparagine at 90^{th} codon
DAB	3,3'-Diaminobenzidine
DiGE	difference gel electrophoresis
DM	degenerative myelopathy
DNA	deoxyribonucleic acid
DPX	distyrene plasticizer xylene
DTT	dithiothreitol
e.g.	example
E40K	substitution of amino acid glutamic acid to lysine at 40^{th} codon
ECL	enhanced chemi-luminescence
EDTA	Ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EMG	electromyography
EPO	erythropoetin
EPR	electronic patient record
ER	endoplasmic reticulum
ESI	electrospray ionisation

fALS	familial amyotrophic lateral sclerosis
FGF-2	fibroblast growth factor-2
FIG4	fig4 homolog gene
FIt3	FMSlike tyrosin kinase 3
FT-ICR	fourier transfom ion cylotron
FTLD	frontotemporal lobar degeneration
FUS	fused in sarcoma/translocated in liposarcoma
g	gram
G	gauge
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
gDNA	genomic DNA
GGT	gamma-glutamyl transferase
GNDF	glial cell-line-derived neurotrophic factor
GSD	German Shepherd dog
GSDM	German Shepherd dog myelopathy
H&E	hematoxylin and eosin
HCl	hydrochloric acid
HGF	hepatocyte growth factor
Hp	haptoglobin
HRP	horseradish peroxidase
HVR2	hypervariable region 2
ICP	inductively couple plasma
IE	idiopathic epilepsy
IEF	isolectric focusing
IgG	immunoglobulin G
IHC	immunohistochemistry
kDa	kiloDalton
kV	kiloVolt
l	litre
LBHI	Lewy body-like hyaline inclusions
LC	liquid chromatography
LMN	lower motor neuron
M	molar
m/z	mass-to-charge
M±SD	mean ± standard deviation

mA	milliampere
MALDI	matrix assisted laser desorption/ionisation
mAU	milliarbitrary unit
MBP	myelin basic protein
MCI	mild cognitive impairment
MCP-1	monocyte chemoattractant protein-1
MEN	meningoencephalitis
MES	2-(N-morpholino)ethanesulfonic
mg	milligram
min	minute
Mkr	marker
ml	millilitre
mm	millimetre
mM	millimolar
MMA	methylmalonic acid
mmol	millimole
MMP-2	matrix metalloproteinase-2
MMP-9	matrix metalloproteinase-9
MNCV	motor nerve conduction velocity
MRI	magnetic resonance imaging
mRNA	messenger RNA
MS	mass spectrometry
MUNE	motor unit number estimation
N	sample size
NaCl	sodium chloride
ng	nanogram
nm	nanometer
NMR	nuclear magnetic resonance
NSE	neuron-specific enolase
OPTN	optineurin gene
PAP	peroxidase-anti peroxidase
PC2	prohormone convertase 2
PCR	polymerase chain reaction
PD	Parkinson's disease
PDI	protein disulphide isomerase

PEDF	pigment cell-derived factor
PHA	phytohaemaglutinin
pmol	picomole
PMSF	phenylmethanesulfonyl fluoride
PPMS	primary progressive multiple sclerosis
PRRS	pig reproductive and respiratory syndrome
p-tau	phosphorylated tau
PTM	post-translational modification
PWC	Pembroke Welsh Corgi
RANTES	regulated on activation, normal T cell expressed and secreted
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolutions per minute
RT	reverse-transcriptase/reverse transcription
sALS	sporadic amyotrophic lateral sclerosis
SCD	sub-acute combined degeneration
SDO	spinal dural ossification
SDS	sodium-dodecyl-sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SELDI	surface-enhanced laser desorption/ionisation
SETX	senataxin gene
*SOD*1	superoxide dismutase 1 gene (human)
SOD1	superoxide dismutase 1
*Sod*1	superoxide dismutase 1 gene (animal)
*SPG*11	spastic paraplegia 11 gene
T18S	substitution of amino acid theorine to serine at 18^{th} codon
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TBS	Tris-buffered saline
TDP-43/TARDBP	TAR-DNA binding protein
TIMP-1	TIMP metallopeptidose inhibitor-1
TIMP-2	TIMP metallopeptidose inhibitor-2
TOF	time-of-flight
t-tau	total tau

T-TBS	Tris-buffered saline with Tween 20
TTR	transthyretin
U	unit
UGSAH	University of Glasgow Small Animal Hospital
UK	United Kingdom
UMN	upper motor neuron
UPR	unfolded protein response
USA	United States of America
V	voltage
VAPB	vesicle-associated membrane protein gene
VDAC1	voltage-dependent anion channel 1 protein
VDMB	Veterinary Medical DataBases
VDS	Veterinary Diagnostic Services
VGF	neurosecretory protein VGF
WT	wild type
xg	centrifugal force in gravity
α-TTP	α-tocopherol transfer protein
ε-EACA	epsilon amino-caproic acid

1 Introduction

1.1 Nosology of Canine Degenerative Myelopathy (DM)

1.1.1 Establishment of Disease Characteristics

The occurrence of chronic progressive ataxia in aging large breed dogs has been recognised for many years. The initial description of a cluster of clinical findings, consistent with what is now thought of as degenerative myelopathy (DM), was first described by Averill in 1973. DM was characterised as a syndrome of progressive pelvic limb ataxia with asymmetrical weakness, commonly affecting older German shepherd dogs (GSD) (Averill, 1973). DM had been formerly attributed to the effects of ossifying pachymeningitis or spinal dural ossification (SDO), although the original reference to this speculation is not found. SDO has been characterised by the presence of elliptical osseous plaques on the internal surface of the dura mater and is reported most frequently in large breed dogs that are middle aged or older (Hoerlein, 1978). These osseous dural plaques can occur at any location in the vertebral column but are most commonly found along the ventral surface of dura mater with higher prevalence at the cervical and lumbar segments of the spinal cord (Hoerlein, 1978). The plaques may coalesce to develop a hard tube, which had been speculated to relate with the abnormal gait and pelvic limb weakness seen in aging large breed dogs (Morgan, 1969). In one study, the occurrence of SDO was reported in 74 out of 114 dogs (65%) over two years of age, however, of the cases affected by SDO only 23% had chronic signs of abnormal gait and pelvic limb weakness. No positive relationship was established between SDO and the pelvic limb signs. The distribution of the lesion in dogs examined was greater from third cervical to first thoracic (C3-T1) and from first lumbar to sixth lumbar (L1-L6) spinal cord. Such lesions could potentially compress nerve roots, and most likely affect spinal nerves forming the brachial and lumbosacral plexuses rather than thoracolumbar spinal cord. This finding was supported by a pathology study of seven GSD, which concluded that the occurrence of SDO in dogs was not found to correlate with symptoms observed in GSD and histopathological changes in the spinal cord (Averill, 1973).

The condition was referred as “Degenerative Myelopathy” (DM) based on the clinical and pathologic findings in aging GSDs (Averill, 1973). The pathological study of seven dogs revealed massive degeneration of axons and myelin with astrocytosis and astrogliosis, which occurred at various segments of the spinal cord, however no brain abnormalities were detected. The lesions were most extensive in mid thoracic region. There was no

consistent abnormality detected in nerve roots except in two dogs, in which there was the loss of individual axons and myelin sheath in thoracic dorsal nerve root. In addition, spondylosis, intervertebral disc protrusion and SDO were also described in several of the dogs examined, however localisation did not correlate with the clinical signs observed. The range of age of affected dogs in this study was 6 to 11 years old. The neurologic abnormalities were restricted to the pelvic limbs, with a variable degree of paraparesis and weakness, and increased patellar reflexes. Thoracic limb was unaffected in all dogs examined.

Griffiths and Duncan subsequently published a report on DM in 1975, focusing on the correlation between clinical, electrophysiological and pathological features of the disease. In general, the findings in this study supported Averill's findings, except the involvement of dorsal nerve root abnormalities was greater in subjects examined. Therefore, the term "Chronic Degenerative Radiculomyelopathy" (CDRM) was proposed (Griffiths and Duncan, 1975). Due to inconsistency of the dorsal root lesions in the subsequent study and the strong breed predisposition, the term "German Shepherd Dog Myelopathy" (GSDM) was later suggested (Braund and Vandevelde, 1978). In light of the various terms proposed for the disease, in this account, it will be referred to as DM.

1.1.2 Breed Predisposition

DM is commonly associated with large breed dogs and their crosses however it has been recognised to occur in older GSDs. The overall prevalence of DM was reported as 0.19% with specific GSD breed prevalence of 2.01% (Coates et al. 2007). DM has also been reported in several other large breed dogs and their crosses including Irish Setter (Averill, 1973), Collies, Rhodesian Ridgeback, Labrador (Griffiths and Duncan, 1975), Siberian Husky (Bichsel et al. 1983), Chesapeake Bay Retriever (Awano et al. 2009; Long et al. 2009), Boxer (Awano et al. 2009; Miller et al. 2009) and recently Bernese Mountain dogs (Wininger et al. 2011). Affected small or medium sized breeds are uncommon however recent evidence has revealed a substantial number of DM-affected Pembroke Welsh Corgis (PWCs) with a breed prevalence of 0.58% (Coates et al. 2007; March et al. 2009). Other small breeds that have been reported with DM include Cavalier King Charles Spaniels (Hopkins, A., personal communication, 04 April 2012), Wire Fox Terriers (Coates et al. 2007) and a miniature Poodle (Matthews and de Lahunta, 1985). The high prevalence of DM in specific breeds suggests the potential significance of a genetic factor in the

aetiology of DM (Coates et al. 2007). The breed-specific prevalence rates for degenerative myelopathy is summarised in Table 1-1.

Breed	Prevalence rates (%)
German Shepherd Dog	2.01
Chesapeake Bay Retriever	0.83
Rhodesian Ridgeback	0.74
Irish Setter	0.68
Boxer	0.59
Pembroke Welsh Corgi	0.58
Collie	0.38
Mixed Breed	0.15

Table 1-1: The latest breed-specific prevalence rates for degenerative myelopathy in selected dog breeds.
This information is adapted from Coates and colleagues (2007) based on the authors' query to Veterinary Medical DataBases (VMDB) in 2000. The prevalence rates were calculated on the number of dogs presented to veterinary teaching hospitals in North America between 1 January 1990 and 31 December 1999.

1.1.3 Age of Onset and Sex Predominance

DM is traditionally associated with older dogs, yet, there is a substantial variation in the age of onset described from six months (Longhofer et al. 1990) to 15 years (Cherubini et al. 2008). There are a few reports of young GSDs affected by DM, however, the age of onset is usually five years or older, with a mean age of nine years in large breed dogs (Averill, 1973; Griffiths and Duncan, 1975; Johnston et al. 2000; Kathmann et al. 2006). In PWCs, reported mean age of onset is 11 years (Coates et al. 2007).

There is no sex predilection for DM, however there may be a breed effect (Coates et al. 2007). Male GSDs are overrepresented in the majority of the case studies (Averill, 1973; Griffiths and Duncan, 1975; Johnston et al. 2000), but in two studies describing PWCs, a predominance of affected females has been noted (Coates et al. 2007; March et al. 2009).

1.1.4 Aetiology and Pathogenesis

A number of studies have considered potential aetiology, however, the underlying cause of DM has remained elusive until the recent breakthrough of a *Sod*1 mutation was discovered in DM (see 1.2, page 40). Prior to the genetic identification, DM had been associated with several aetiologies including nutritional deficiencies and auto-immune defects.

As discussed in 1.1.1 (page 24), DM was previously ascribed to the occurrence of SDO, the multiple osseous plaques in the dura mater that were speculated to cause pressure on the nerve roots leading to the development of the paraparesis (Morgan, 1969). However, Morgan (1969) and Averill (1973) had demonstrated that the occurrence of SDO did not correlate with the pathological changes seen in paraparesic dogs and suggested SDO was likely to be incidental. Averill (1973) also discounted the idea of vascular insufficiency causing degenerative changes in the spinal cord based on the anatomical distribution of the lesions found in DM cases. The distribution of ischemic lesions is reflected by the location of the affected vessels and is related to focal and asymmetric abnormalities. The central artery of the spinal cord is most commonly affected, leading to severe necrosis of the gray matter columns that occasionally extends to the white matter. Neither of these lesions was found in the dogs examined in Averill's study. In addition, the clinical onset of vascular problems is rather acute in nature and briefly progressive, which is not consistent with DM.

In 1973, Averill explored the similarities of DM with the myelopathy associated vitamin B12 deficiency in humans, also known as sub-acute combined degeneration (SCD). Although differences between these conditions exist, they involve progressive and gradual degeneration of white matter in thoracic segments. In SCD, the patterns of the pathological features are usually patchy or multifocal instead of topographically continuous as described in DM. The neurologic manifestation of SCD is attributed to the defective methylation of methylmalonic acid (MMA) (Baik and Russell, 1999). The measurements of serum B12 levels in six DM-affected GSDs were sub-normal in three dogs (Williams et al. 1984). In the same study, Williams and others (1984) examined the association of hypovitaminosis B12 with the occurrence of small intestinal disease in the affected GSDs. Jejunal biopsies in the affected dogs demonstrated no histological changes, however marked enzyme elevations were detected in the jejunal mucosa. Overgrowth of bacteria was also observed in duodenal juice cultures of affected dogs ($9.7x10^4$/ml – $2.3x10^7$/ml compared to controls $>1.9x10^4$/ml). Based on these findings, the authors speculated that

the enteropathy observed in DM-affected dogs might be responsible for the lesion development due to malabsorption of essential nutrients.

An association between DM and vitamin E deficiency causing myelopathy in human has been described in the literature, which is known as ataxia associated vitamin E deficiency (AVED) (Muller, 1986). In human, AVED has been associated with mutations in the gene of α-tocopherol transfer protein (α-TTP), impairing the α-TTP functions, leading to failure in retaining α-tocopherol from dietary vitamin E (Ouahchi et al. 1995). The low α-tocopherol levels in the blood lead to an accumulation of free radical oxygen that eventually contributes to the neuronal degeneration in the spinal cord, brain stem and peripheral nerves (Muller, 1986; Imounan et al. 2012). Williams and colleagues (1985) investigated the concentrations of serum α-tocopherol in healthy controls and DM-affected dogs. In their study, the serum levels of vitamin E in DM affected group (4.4±1.2mg/l) were slightly lower compared to controls (14.1±2.1mg/l), suggesting that the enteropathy they saw in the earlier study may have been responsible for the malabsorption of the α-tocopherol and subsequently development of DM (Williams et al. 1985). Another study had measured the serum α-tocopherol concentrations in 25 affected GSDs and 46 unaffected dogs (20 GSDs and 26 other breeds) (Johnston et al. 2001). The mean of the serum α-tocopherol levels in affected GSDs were significantly higher (46.4µmol/l) than the unaffected dogs (34.2µmol/l), however not significantly higher than unaffected GSDs (37.3µmol/l). Sequencing of the canine α-TTP cDNA revealed no differences in either nucleotide or predicted amino acid sequences (Fechner et al. 2003). In addition, no significant difference was found in the level of α-TTP mRNA in affected GSDs and controls. Therefore, these findings strongly suggest that abnormal α-TTP function is not a contributing factor to DM development.

Griffiths and Duncan (1975) considered that DM pathology was indicative of a “dying-back” disease or distal axonopathy. This was speculated due to the long nerve fibres in the large breed dogs that may increase the susceptibility to the dying-back degeneration (Griffiths and Duncan, 1975). Braund and Vandevelde (1978) argued that the distribution of DM lesions did not fit the classic dying-back pattern. The classic dying-back lesions are typically symmetrical and often occur due to secondary metabolic or toxic insults that interrupt axonal transport (Spencer and Schaumburg, 1978), which initially occurs in the distal or peripheral axonal portion and gradually spreads towards the proximal axonal portion (Cavanagh, 1964). However lesions in DM-affected dogs predominantly involve

asymmetrical white matter tract degeneration and are not restricted to particular nerve fibres as typically observed in dying-back process (Braund and Vandevelde, 1978; Johnston et al. 2000). In addition, the severity of the DM lesions in thoracic segments is suggestive of the selective vulnerability of the thoracic spinal cord (Braund and Vandevelde, 1978; Johnston et al. 2000).

In 1980, a different research group considered the role of the immune system in DM (Waxman et al. 1980b). They evaluated the peripheral lymphocyte response to mitogens in seven DM-affected dogs and had indicated marked impairment in the proliferative response to thymus-dependant mitogens; concanavalin A (Con A) and phytohaemaglutinin P (PHA) by peripheral blood leukocytes. In contrast, lymph node and splenic leukocytes from affected dogs developed normal response to the thymus-dependant mitogens, which leads to speculation of an agent in the peripheral blood that was causing the suppression. In a follow-up investigation using the same affected GSD population, the impaired proliferative responses were associated with an aberrant suppressor cell in the peripheral blood that may be mediated by the release of prostaglandins (Waxman et al. 1980a). The authors speculated that the suppressor cells could be activated secondarily by the host leading to the autoimmune event in DM, however this hypothesis remains unproven.

The hypothesis of an immune-mediated cause was further investigated, evaluating the distribution of immunoglobulin G (IgG) and complement component 3 (C3) in the spinal cords of five DM-affected dogs (Barclay and Haines, 1994). Immunohistochemistry analyses on spinal cord tissues from various regions were examined in six dogs including one normal dog. In this study, increased IgG and C3 staining were observed in DM-affected dogs, associated with the areas of increased vascularisation proximal to the DM lesions. In addition, there were extra-vascular deposits of IgG and C3 along the peripheral edges of ventral and dorsal funiculi, which corresponded closely with the areas of myelin loss. These IgG and C3 depositions were also observed in regions without lesions or vascularisation. Spinal cord tissue from a normal dog demonstrated increased staining in the regions associated with blood vessels, however no specific staining was detected in other tissue areas (Barclay and Haines, 1994). This study has indicated the possibility of immune-mediated destruction in DM pathogenesis, however there is no conclusion on the specific antigens and whether the antigens that triggered the immune-mediated destruction were exogenous or endogenous remains undetermined.

DM has been speculated as a disease with a complex aetiology, potentially contributed to by several causes, however the high incidence of DM in a specific breed undoubtedly indicate a genetic basis. Braund and Vendevelde in 1978 were the first speculating the involvement of genetic factor in DM due to strong predisposition of GSD. This concept was not further pursued until an allele in the hypervariable region 2 (HVR2) of the *DLA-DRB*1 was considered as a candidate gene based on speculation that DM was an orthologue of primary progressive multiple sclerosis (PPMS) (Clemmons et al. 2006). This allele *1101J of *DLA-DRB*1 was claimed to be homozygous in DM-affected GSDs and heterozygous in healthy GSDs (Clemmons et al. 2006). However, in another study, a full sequence analysis on the three homozygous GSDs failed to duplicate the previous genotyping results published by Clemmons and others (2006) and did not support the presence of allele *1101J in the affected GSDs (Clark et al. 2008). Therefore, the authors of this study concluded that the presence of mutant allele *01101J could not be used to predict DM.

Coates and colleagues (2007) pursued the concept of a genetic basis on DM using pedigree information from affected-PWCs, demonstrating a strong familial relationship from one large family of PWCs with 27-affected individuals. The strong possibility that the aetiology of DM has a significant genetic component has driven collaborative studies, which have been recently rewarded. Significant progress in the understanding of the basis of DM has been made with the confirmation that a mutation in superoxide dismutase (*Sod*1) gene has been revealed in selected dog breeds affected with DM (Awano et al. 2009). The result of this study also raises the possibility that DM is a potential animal model for amyotrophic lateral sclerosis (ALS) in man (see 1.4, page 50). Details of the involvement of the genetic cause in DM will be further discussed in 1.2, page 40.

1.1.5 Pathological Features

1.1.5.1 Spinal Cord Pathology

Confirmation of a specific diagnosis remains at the level of histopathological examination of the spinal cord. No consistent gross lesions of the adnexal structures of the central nervous system (CNS) have been reported. Incidental lesions such as SDO and spondylosis are common however they do not associate specifically with the histopathological features reported in DM (Averill, 1973; Johnston et al. 2000).

The spinal cord pathology described in dogs affected by DM is consistent with a non-inflammatory axonal degeneration and consequent demyelination (Averill, 1973; Griffiths and Duncan, 1975; Braund and Vandevelde, 1978; Coates and Wininger, 2010). Extensive degeneration of myelin and axons have been reported in ascending and descending tracts of the white matter with the presence of astrocytosis and astrogliosis (Averill, 1973; Griffiths and Duncan, 1975; Coates et al. 2007). Lesions are observed at all levels of the spinal cord but are most extensive in the middle to caudal thoracic region tapering cranially and caudally along the spinal cord (Griffiths and Duncan, 1975; Johnston et al. 2000). The degenerative changes are more prominent in lateral funiculus, particularly affecting the corticospinal and rubrospinal tracts. Lesions in dorsal funiculus tend to localise in the fasciculus gracilis. In addition, degenerative changes in ventral funiculus have been reported, which consistently found around the ventromedian fissue. There have been no consistent abnormalities found in dorsal nerve root (Johnston et al. 2000), although severe axonal degeneration and loss of myelin sheath in this region was described in early studies (Averill, 1973; Griffiths and Duncan, 1975). Macrophages are occasionally observed in areas of axonal and myelin debris, indicating myelin fragmentation and phagocytosis, which is suspected to be a secondary response to the degenerative process (Averill, 1973; Coates et al. 2007). Lesions in grey matter are usually mild, with astrogliosis and chromatolysis reported in the intermediate and dorsal grey matter (Clark's column) of the caudal thoracic and lumbar spinal cord (Averill, 1973; Johnston et al. 2000). Such abnormalities in other areas of grey matter are rarely described (Griffiths and Duncan, 1975; Johnston et al. 2000).

Awano and others (2009) re-evaluated the spinal cord pathology based on the identification of a *Sod*1 mutation in DM-affected dogs (see 1.2, page 40). Immunostaining using an antibody against SOD1 protein has indicated the presence of SOD1 cytoplasmic inclusion bodies, characterised as well-defined dark clumps (Awano et al. 2009). In a recent study, double fluorescent-immunostaining of thoracolumbar spinal cords from DM-affected dogs has revealed co-localisation of protein disulphide isomerise (PDI) with SOD1 cytoplasmic inclusions (Long et al. 2012). These pathology findings were reported to be similar to those found in ALS patients and transgenic models expressing mutant human *SOD*1 (Atkin et al. 2008; Honjo et al. 2011).

1.1.5.2 Brain Pathology

Early reports characterised the pathological features of DM highlighting neuronal and axonal degeneration restricted to the spinal cord (Averill, 1973; Griffiths and Duncan, 1975). However, Johnston and others (2000) described previously unrecognised abnormalities in specific nuclei in the brain. Abnormalities were consistently detected in the red nucleus (origin of the rubrospinal tract), although the lesions were also described in the lateral vestibular, dentate and fastigial nuclei (Johnston et al. 2000). Within these nuclei, the abnormalities observed include chromatolysis, occasional neuronophagia and gliosis. Electron microscopy of the affected red nucleus revealed enlarged axons containing a high number of disorganised neurofilaments, loss of Nissl substance and clumping of numerous membranous organelles (Johnston et al. 2000). In addition, nerve fibres undergoing Wallerian degeneration were also detected within the red nucleus.

1.1.5.3 Peripheral Nerve and Muscle Pathology

Lower motor neuron (LMN) signs in DM do not become evident until later in the disease progression (Coates et al. 2007; Coates and Wininger, 2010). Lesions at the dorsal nerve root have been reported however were found to be inconsistent between studies (Griffiths and Duncan, 1975; Johnston et al. 2000). Other pathological features of the peripheral nerve and muscle are rarely reported in DM as most of the affected dogs are euthanised before manifesting the LMN signs of the later stage. However, a recent study has documented neuromuscular abnormalities in DM manifesting LMN signs, demonstrating muscle atrophy consistent with denervation and demyelination of peripheral nerves (Shelton et al. 2012). Marked variability in myofibre size with large groups of atrophic fibres was detected in DM-affected Boxers with LMN paraplegia. Fibre loss and myelin ovoids were evident within the distal intramuscular nerve branches which were consistent with Wallerian degeneration. Extensive loss of nerve fibres and endoneurial fibrosis were also obvious in the peroneal nerve. At later disease stages, generalised muscle atrophy was prominent in affected PWCs with LMN tetraplegia and brain stem signs. These abnormalities usually were not found in early stages although a study on mild stage DM in the affected Boxers did demonstrate occasional, small groups of atrophic fibres in biceps femoris and gastrocnemius muscles (Shelton et al. 2012).

1.1.6 Clinical Spectrum

The classification scheme of DM clinical signs, diagnostic methods and recommended management for DM cases are summarised in Figure 1-1. The details of the clinical sign of DM have been well documented (Averill, 1973; Griffiths and Duncan, 1975; Coates and Wininger, 2010). Non-painful, gradually progressive upper motor neuron (UMN) paresis with ataxia and weakness of pelvic limbs are the key features of DM. The onset is insidious with the predominant age of onset around six to nine years. Scuffing with the two middle toes of one or both pelvic limbs is commonly seen during early disease onset, which progresses to wearing and bleeding claws (Johnston, 1998; Cherubini et al. 2008). Subsequently, they develop problems with climbing/going down the stairs, misjudged distances and hypermetria (Johnston et al. 2001). Both pelvic limbs are usually involved although asymmetry of signs is frequently reported. Pelvic limbs crossing during walking and swaying movement of the pelvis are also apparent at this stage (Lorenz and Kornegay, 2004). Neurogenic muscle atrophy over the pelvic limbs and paresis occur with time that eventually leads to non-ambulatory paraparesis. Euthanasia is usually elective and related to disease progression, often as the circumstances develop to non-ambulatory paraparesis.

Neuroanatomic localization in the early stage of the disease suggests lesion localisation between T3 and L3 of the spinal cord. On neurological examination, conscious proprioception including reflex stepping and sway tests were affected either uni- or bilateral depending on the severity of the disease (Griffiths and Duncan, 1975). Spinal reflexes testing are suggestive of UMN dysfunction. In many cases, patellar reflex is normal although exaggeration to clonic reflex may be seen in affected-dogs. LMN sign of patellar reflex (hyporeflexia) can be observed in some cases. However, this could also be related to normal age-dependent decline in patellar reflex magnitude (Coates and Wininger, 2010). Flexor or withdrawal reflex are normal, crossed extensor reflex may be present, and if it is present, is usually suggestive of chronic UMN dysfunction. During the latter stage of disease, signs of LMN disease such as flaccidity due to denervation, hyporeflexia of patella and withdrawal become more apparent. Dogs with advanced DM will exhibit LMN signs including severe, neurogenic muscular atrophy, hyporeflexia and flaccid weakness (Awano et al. 2009). Spinal reflex examination reveals hyporeflexia of patella, withdrawal and cranial tibial reflexes. The paresis also becomes more symmetric as the disease progresses, which eventually will ascend to the thoracic limb (Averill, 1973; Matthews and de Lahunta, 1985; Kathmann et al. 2006) and followed by tetraplegia as well as generalised muscle atrophy. Swallowing difficulty and inability to bark has also

been reported in PWC-affected cases (Coates et al. 2007). Urinary and fecal incontinence too has been reported in long-standing cases (Kathmann et al. 2006; Coates et al. 2007).

The disease progression in affected dogs is not constant and clinical signs may have been stabilised before reaching the stage of acute deterioration (Figure 1-1). Most of the affected dogs will progress to non-ambulatory paraparesis within six to nine months from the onset of clinical signs and are usually euthanised at this stage (Coates and Wininger, 2010). The mean duration of clinical signs was reported to be longer in PWC-affected dogs with an average of 19 months (Coates et al. 2007; Awano et al. 2009; March et al. 2009). If the dog is not euthanised, the clinical signs will evolve to LMN paraplegia and muscle atrophy occurring between 9 and 18 months post onset. Involvement of thoracic limb will be apparent between 14 to 24 months followed by LMN tetraplegia and brainstem abnormalities within 24 to 36 months. The natural cause of death in DM is not determined as most of the dogs are euthanised when they become non ambulatory paraparetic, however respiratory difficulty may be observed at the end stage which could potentially lead to respiratory failure (Vasquez, 2011).

1.1.7 Clinical Diagnosis

The specific diagnosis of DM in a clinical environment is challenging due to the spectrum of clinical signs that are common to many diseases and due to the lack of a specific diagnostic test. As DM predominately affects older dogs which are prone to orthopaedic and neurologic problems, the presence or potential presence of these other conditions may affect the interpretation of the neurological examination (Coates and Wininger, 2010). The most common neurological disorder of older large breed dogs is Hansen type II disc disease, although in the chondrodystrophic breed such as PWC, Hansen type I is more significant. Other differential diagnoses or conditions that may mimic DM include degenerative lumbosacral syndrome, spinal cord neoplasia, and degenerative joint disease (Cherubini et al. 2008; Coates and Wininger, 2010).

Clinical diagnosis of DM relies on the history and clinical findings with supportive findings on investigation helping to eliminate other potential causes of the individual's clinical problems. Routine haematology and biochemistry consistently reveal no abnormalities. Cerebrospinal fluid (CSF) analysis in DM affected dogs has been limited and has been used to exclude other CNS problems. CSF analysis is often unremarkable, though may demonstrate albuminocytological dissociation (Cherubini et al. 2008).

Magnetic resonance imaging (MRI) and computerised tomography (CT) scan are frequently inconclusive in diagnosing DM but effective in ruling out other CNS diseases (Cherubini et al. 2008). In the early stage of the disease, electrophysiological studies such as electromyography (EMG) and motor nerve conduction velocities (MNCV) have failed to detect abnormalities, implying the lack of involvement of peripheral nerves and motor fibres early on in the disease (Griffiths and Duncan, 1975). However, in the latter stages EMG in affected dogs has demonstrated multifocal spontaneous activity in the distal limb appendicular musculature (Awano et al. 2009; Coates and Wininger, 2010) with fibrillation potentials and sharp waves being most commonly recorded. The compound muscle potentials (M wave) in the tibial and ulnar nerves show temporal dispersion, decreases in amplitude and reduced velocities (Awano et al. 2009).

1.1.7.1 Genetic Analysis

A genetic cause has been speculated in the pathogenesis of DM based on the epidemiology of the disease, as discussed in 1.1.4, page 30 (Braund and Vandevelde, 1978; Coates et al. 2007; Clark et al. 2008). This has been confirmed by a recent study using a genome wide mapping association, which revealed a point mutation in exon two of the canine *Sod*1 gene, predicting G to A nucleotide transition at 118th nucleotide (see 1.2, page 40). Homozygosity of the mutant allele in the *Sod*1 gene (118G>A) has been considered a risk factor for the development of DM (Awano et al. 2009; Vasquez, 2011).

Genotype analysis based on this mutation has been developed and is commercially available as a diagnostic test for DM cases (Awano et al. 2009). The use of the *Sod*1 genotyping test for DM in conjunction with clinical investigations has significantly increased the index of suspicion of DM and improves the disease prediction. However, although the *Sod*1 mutation is strongly associated with DM, this mutation is also found in a proportion of the asymptomatic dog population (Awano et al. 2009). Therefore *Sod*1 genotyping against 118G>A mutation is not specifically diagnostic for DM.

1.1.8 Attempts at Identifying a Biomarker to Assist Clinical Diagnosis

Successful characterisation of specific and reliable biomarkers for DM would assist the clinical diagnosis and improve the understanding of the disease mechanisms. In addition, such discoveries may aid in the development and evaluation of novel therapies for DM.

There are no specific biomarkers for DM to date, although a genetic marker has been recognised as a risk factor in DM (see 1.2, page 40). In this section, I summarise a number of other avenues that have been explored in DM.

A study was performed to establish CT myelographic characteristics of the thoracolumbar spine in a group of dogs (eight dogs) that were clinically diagnosed as DM. Characteristics of the CT myelography that were observed in higher frequency than the clinically normal dogs included spinal stenosis, disc protrusion, focal attenuation of subarachnoid space, spinal cord deformity, small spinal cord and paraspinal muscle atrophy (Jones et al. 2005). However, these characteristics are not specific to DM and there is the possibility that these features are caused by chronic disc protrusion rather than DM. In addition, none of the affected dogs were examined histopathologically, and therefore association between CT myelography characteristics and definitive pathology was not determined.

CSF analysis in DM affected dogs has been consistently unremarkable and is frequently used to eliminate other potential CNS problems. Several studies have evaluated selected CSF proteins as biomarker candidates for DM. A study using commercially available human MBP enzyme-linked immunosorbent assay (ELISA) had detected a significant elevation of myelin basic protein (MBP), a key myelin protein, in lumbar cistern CSF in DM-affected GSDs (3.13±0.46ng/ml) compared to CSF obtained from cisterna magna (0.70±0.06ng/ml) in DM-affected GSDs and from both cisterna and lumbar samples from control dogs (Oji et al. 2007). This observation suggested the presence of active demyelination lesion in DM that may occur secondarily to the axonal degeneration. The level of MBP in CSF has been a useful indicator for demyelinating disorders in human (Whitaker, 1998; Ohta and Ohta, 2002) and therefore may also prove to be valuable marker for DM. This study has indicated the potential utility of the human MBP ELISA as a supportive diagnostic tool for DM in the clinical environment, however further validation of MBP in DM and other canine neurological disorders is required as this protein may not be specific to DM.

Coates and others (2007) characterised the clinicopathological findings of a familial DM in PWCs (see 1.1.4, page 30) at the same time measuring the concentrations of 8-isoprostane in DM-affected CSF, which is an oxidative metabolite that has been considered as a reliable and stable marker for oxidative stress in human neurological disorders (Greco et al. 1999; Montine et al. 1999; Montuschi et al. 2004). There was no significant difference in the CSF level of 8-isoprostane between affected and normal dogs. However, the authors

have claimed that the CSF level of 8-isoprostane collected from cisterna magna may underestimate the effect of spinal cord damage and a significant result may be obtained if the protein concentrations were measured using lumbar cistern CSF (Coates et al. 2007).

Previous studies have demonstrated the potential involvement of an autoimmune event in the pathogenesis of DM (1.1.4, page 29). Therefore, a study has measured the concentrations of total protein, IgG and total protein/IgG ratio on six DM-affected GSDs to determine whether there was evidence of intrathecal IgG synthesis that may support the altered immune response in DM. There was no significant difference in IgG or total protein concentrations detected between the affected and control groups (Kamishina et al. 2008). Isoelectric focusing (IEF) followed by immunofixation using canine CSF revealed the presence of oligoclonal bands in four DM-affected GSDs, which is indicative of intrathecal IgG synthesis in these cases. However, the significance of these bands was questionable since a similar band was also detected in two control samples, and therefore it is unlikely to be specific for DM.

Following the establishment of genetic commonality in DM and ALS, a recent study has developed a motor unit number estimation (MUNE) technique (Vasquez, 2011), which is a common electrophysiology method that is used to monitor ALS progression (Boe et al. 2007; Shefner et al. 2007). This technique was purposely developed to aid in the characterisation of advanced stage DM with chronic LMN signs. The preliminary ranges of MUNE in clinically normal dogs have been established. The longitudinal studies monitoring the lower motor neuron loss in DM-affected dogs is currently ongoing (personal communication, Coates. J.R, May 2012).

1.1.9 Treatment

To date, there is no specific therapeutic modality for specific treatment of DM nor has strong evidence of positive effects of proposed symptomatic treatment been presented. DM was hypothesised as a immune-mediated neurodegenerative disorder, therefore administration of immunosuppressive drugs had been suggested. Glucocorticoids, cyclophosphamide and azothioprine were used in DM cases, which were speculated to slow down the rate of deterioration in affected dogs, however none of these drugs was shown to have a positive effect on disease progression (Clemmons, 1992). An anti-protease agent, epsilon amino-caproic acid (ε-EACA) also had been claimed to help in slowing the degeneration process (Clemmons, 1992). However, evaluation of the long term efficacy of

ε-EACA and N-acetylcysteine in combination with exercise and vitamin B, C and E supplements in affected dogs did not yield promising benefit in either slowing nor improving the condition (Polizopolou et al. 2008).

Physical rehabilitation or physiotherapy has been traditionally recommended in DM cases (Kathmann et al. 2006). Kathmann and colleagues (2006) investigated the effect of long term and intensive physiotherapy in 22 affected dogs, performed by the owners at the time when the presumptive diagnosis was made until the time of euthanasia. All owners had been given careful instructions on how to perform adequate physiotherapy and follow up information was obtained by telephone call. In this study, affected dogs that had received intensive physiotherapy (gait exercise 3-5 times/day, massage and passive joint movement 3 times/daily, or daily hydrotherapy) had longer survival time (mean survival time = 255 days) compared to dogs with moderate (gait exercise 3 times/day, hydrotherapy or massage once a week; mean survival time = 130 days) or no physiotherapy (mean survival time = 55 days), suggesting conservative management may have been beneficial in improving the quality of life in the affected dogs although did not prevent the inevitable outcome.

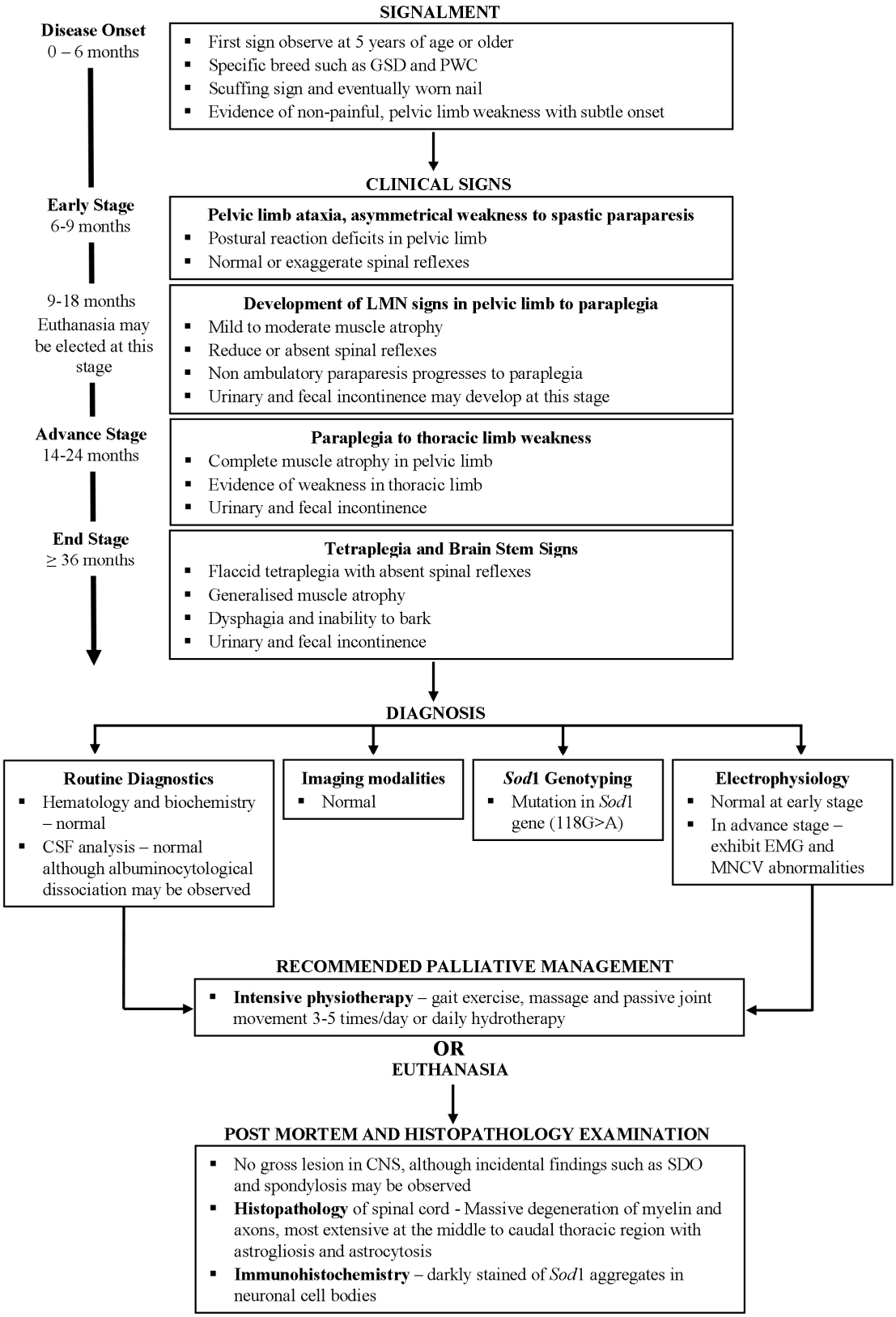

Figure 1-1: Diagrammatic representation of the clinical signs, complete diagnostic work-up with findings, and recommended palliative management in DM cases.

1.2 The Identification of 118G>A Superoxide Dismutase 1 *(Sod1)* Gene Mutation in DM: A Significant Breakthrough

Many studies have investigated the potential aetiologies that may underlie the neurodegeneration in DM as discussed in 1.1.4 (page 27). High incidence of a disorder in a specific breed implies a genetic contribution to the aetiology of the disease (Nicholas, 2003). However, this speculation in DM was not substantiated until a familial relationship was established in DM-affected PWCs, implying a significant association of DM with genetic cause (see 1.1.4, page 30) (Coates et al. 2007).

A significant breakthrough was achieved in the understanding of the genetic basis in DM, with implication of a mutation in *Sod*1 gene in affected dogs, which is comparable to the *SOD*1 mutations in human ALS (Awano et al. 2009). This mutation occurs at the 118th nucleotide that predicts G to A transition in exon two (118G>A), resulting in E40K missense mutation. Genome-wide association mapping in 38 affected and 17 control PWCs identified the strongest association in chromosome 31 (CFA31) containing the canine *Sod*1 gene. Representatives of five DM-affected breeds; PWC, GSD, Boxer, Rhodesian Ridgeback, and Chesapeake Bay Retriever were sequenced for the mutant allele A in the *Sod*1 gene, and demonstrated a significant association between the DM phenotype and homozygosity of the A allele. Ninety-six percent (96 out of 100 dogs) with presumptive diagnosis of DM were confirmed as homozygotes (A/A), however the homozygous genotype was also detected in 34% of control dogs. Furthermore, the percentage of homozygote individuals was found to be higher within certain breeds of the control group; PWC (74%) and Boxer (67%) compared to GSD (25%), Rhodesian Ridgeback (15%) and Chesapeake Bay Retriever (39%). None of the control dogs displayed clinical characteristics of DM. Heterozygous genotype (A/G) was reported in 32% of the control group, however found in a very low percentage of the DM group (2%). These heterozygote individuals are potential carriers that can pass the mutant *Sod*1 gene to their offspring. Two-percent of the affected and 34% of control dogs were also confirmed to be harbouring the normal or wild type *Sod*1 gene (G/G). Immunohistochemical analysis using anti-SOD1 antibody in seven DM-affected dogs revealed well-defined dark brown focal clumps in neuronal cell bodies which may be suggestive of SOD1 protein aggregates. No positive staining was observed in control dogs. The genotype-phenotype correlation in

heterozygotes is unclear however, immunohistochemical analysis on asymptomatic heterozygotes revealed diffuse light staining in the neuronal cell bodies, which may reflect a subclinical condition (Coates and Wininger, 2010).

Subsequent to the identification of the *Sod*1 mutation in DM, a retrospective study investigating the prevalence of 118G>A *Sod*1 mutation in a referral population of GSD in United Kingdom, demonstrated that 76% of the affected GSDs and 24% of control GSDs were homozygous for the mutant A allele (Adams et al. 2010). The percentage of heterozygous population in this study was found to be higher, 33% compared to 2% in the previous study (Awano et al. 2009). GSDs with a wild type gene was reported as 14% and 43% in affected and control GSDs respectively.

The genetic observations in these studies match the characteristic of an incomplete penetrance and the disease is most likely to be inherited in an autosomal recessive manner (Awano et al. 2009). Most of the ALS cases associated with *SOD*1 mutations are inherited through autosomal dominance with high penetrance, however pedigrees with recessive traits with incomplete penetrance, D90A (aspartic acid to alanine) have been reported in some families (Andersen et al. 1996; Andersen, 2006). The D90A *SOD*1 mutation has been described to have a slower disease progression with initial onset involving the lower limbs, which is more likely to resemble the E40K missense mutation in DM (see 1.3.3, page 46).

A genotyping analysis based on the presence of 118G>A *Sod*1 mutation has been developed by Professor Joan R. Coates and her group from University of Missouri, and is currently being used as a routine diagnostic analysis for DM and a screening test for selective breeding programmes. To date, more than 23,000 dogs have been genotyped for this mutation (Coates, J.R., personal communication, May 2012).

1.2.1 Recent Identification of a Novel *Sod*1 (52A>T) Missense Mutation in a Bernese Mountain Dog Affected by DM

In a very recent study, another *Sod*1 mutation has been identified in a Bernese Mountain dog (BMD) affected with DM (Wininger et al. 2011). Sequencing of *Sod*1 gene in this case has revealed a homozygosity of the mutant allele T at the 52nd nucleotide (52A>T) that predicts the substitution of amino acid threonine to serine at the 18th position in the amino acid sequence (T18S). Immunohistochemical staining against SOD1 protein

displayed consistent findings to those harbouring the 118G>A mutation (Awano et al. 2009; Wininger et al. 2011). Although the appearance of SOD1-containing aggregates in this case is most likely considered to be related to the neurodegenerative process, a definitive conclusion can only be made after the relationship of 52A>T *Sod*1 mutation with the clinical and pathological findings is established.

1.3 Amyotrophic Lateral Sclerosis (ALS) and *SOD*1 Mutations

1.3.1 Overview of ALS

Amyotrophic lateral sclerosis or Charcot's disease, is a devastating, fatal neurodegenerative disease, affecting approximately 1 to 3 per 100,000 individuals per year (Jackson and Bryan, 1998; Leigh, 2007). This disease was first described in 1874 by Jean-Martin Charcot, and is characterised as late onset and progressive motor neuron disease resulting from degeneration of UMN and LMN systems (Rowland and Shneider, 2001). It is also reported that ALS is responsible for approximately 1 in 1000 deaths (Andersen, 2006).

ALS occurs in both sporadic and familial forms. Most ALS cases are classified as sporadic cases (sporadic ALS), while 5 to 10% are reported to be familial (familial ALS) (Battistini et al. 2010). Familial ALS cases are commonly inherited through an autosomal dominant pattern with high penetrance, however recessive pedigrees have been described in some families (Khoris et al. 2000; Hand et al. 2001). Mutations in the *SOD*1 gene are the most frequently identified cause of familial ALS and account for 20% of familial ALS cases (Rosen et al. 1993; Ticozzi et al. 2011). Other than the *SOD*1 gene mutation, TAR DNA binding protein (*TARDBP*) and fused in sarcoma/translocated in liposarcoma (*FUS*) have also recently been associated with familial ALS, each contributing 5% of total familial ALS cases (Ticozzi et al. 2011). Pathogenic mutations in seven other genes; amyotrophic lateral sclerosis 2 (*ALS*2), senataxin (*SETX*), spastic paraplegia 11 (*SPG*11), vesicle-associated membrane protein B (*VAPB*), angiogenin (*ANG*), FIG4 homolog (*FIG*4) and optineurin (*OPTN*) account for less than 5% of total familial ALS cases (Ticozzi et al. 2011). Whilst a genetic predisposition is described as a major risk factor in familial ALS, the aetiology of sporadic ALS remains elusive, although a genetic component has also

been attributed to a minority of sporadic ALS cases including *SOD*1 mutations, these accounts for 1-7% of sporadic ALS cases (Jackson et al. 1997; Gellera et al. 2001).

In general, the average age of onset of ALS is between 55 to 65 years of age, although the average onset in familial ALS cases is a decade earlier (Leigh, 2007). Occurrence of ALS when age is less than 25 years is characterised as juvenile form (Ben et al. 1990). Men are more frequently affected than women with a male to female ratio of 3:2, although more recent data has indicated that the ratio is approaching 1:1 (Ticozzi et al. 2011). The classic clinical features of ALS include progressive muscle weakness and atrophy, eventual paralysis and death. Approximately, two third of patients with classic ALS have a spinal form of the disease with first symptoms related to asymmetric focal muscle weakness and wasting, which may start either in the upper or lower limb (Jackson and Bryan, 1998; Wijesekera and Leigh, 2009). Difficulty lifting the upper and lower limbs and clumsiness are the first signs noticed by patients. Cramps and fasciculation may precede weakness however these abnormalities are rarely noticed until the later stage of the disease. Spastic paresis develops gradually after the first symptoms, affecting manual dexterity and gait. In advanced stage ALS most patients develop bulbar signs (dysarthria and dysphagia) and eventually died due to respiratory failure. Disease duration from first onset until respiratory failure is between two to five years (mean 2.5 years), although some patients may have a longer disease duration up to 10 years or more (Cudkowicz et al. 1997; Ratovitski et al. 1999). Urgency of micturition or even incontinence (Leigh, 2007) as well as cognitive impairment (Strong et al. 1996) although uncommon, may occur in a minority of ALS patients during the late stage of the disease. For patients with bulbar onset, the first symptom is always dysarthria followed by dysphagia within weeks or months (Leigh, 2007). Cranial nerve abnormalities such as facial weakness and tongue atrophy may be observed in bulbar onset patients (Leigh, 2007; Wijesekera and Leigh, 2009). The limb abnormalities may develop simultaneously with the bulbar symptoms and mostly occur within one to two years after the first signs.

The diagnosis of ALS largely depends on extensive patient history, recognition of clinical characteristics and supportive investigations (Wijesekera and Leigh, 2009). El-Escorial criteria for ALS were approved and have been revised over the years to improve early diagnosis and are currently being used as a standard method for diagnosing ALS (Brooks, 1994; Brooks et al. 2000). Genetic screening has become part of the diagnostic protocol to determine the genetic risk in suspected ALS patients (Siddique et al. 1991) and is also

being utilised for presymptomatic testing in potential familial ALS individuals (Fanos et al. 2004). However, in many cases diagnosis takes over a year following disease onset which represents one-third of the disease duration (Leigh, 2007). Such a delay in diagnosis is generated from misdiagnosis and difficulties in differentiating ALS from other related disorders with similar clinical characteristics (Leigh, 2007). Therefore, over the past few years, an intensive search for ALS biomarkers has been initiated, with particular interest in characterising an early diagnostic biomarker to support the diagnosis of ALS (Bowser et al. 2006). Details on the development of biomarkers in ALS are described in 1.7, page 68.

The major pathological features of ALS include degeneration and loss of motor neurons with astrocytic gliosis and the presence of various inclusion bodies in degenerating neurons and glial cells (Hirano, 1996). CNS pathology involves severe loss of pyramidal neurons (Betz's cells) (Hammer, Jr. et al. 1979) in the primary motor cortex, diffuse degeneration of the motor pathways of the corticospinal tract in the lateral and anterior funiculi of the spinal cord (Tandan and Bradley, 1985) and degeneration of brain stem nuclei of cranial nerves V, VII, IX, X and XII (Jackson and Bryan, 1998). Astrogliosis is also a common pathological feature of ALS (Schiffer et al. 1996). Lesions are also described in the peripheral nervous system (PNS); primarily involving axonal degeneration and demyelination of ventral roots particularly in cervical and lumbar regions with milder lesions found in thoracic and sacral regions (Sobue et al. 1981). A reduction in the number of neurons in lumbar dorsal root ganglion have also been reported in a minority of ALS cases (Kawamura et al. 1981). Neurogenic atrophic changes in muscles such as pyknotic nuclei and fibre type grouping are also common in ALS patients (Fidzianska, 1976).

An established hallmark of ALS is the presence of various inclusion bodies in degenerating neurones and surrounding reactive astrocytes (Barbeito et al. 2004). The most common and specific type of inclusion bodies is the ubiquitinated inclusions in brain and spinal cord, which can be seen in up to 95% of ALS cases (Leigh et al. 1988). These inclusion bodies are characterised as Lewy body-like inclusions and Skein-like inclusions (Hirano, 1996). Lewy-body like hyaline inclusions (LBHIs) and astrocytic hyaline inclusions (Ast-His) containing SOD1 antigen are more commonly seen in ALS patients with *SOD*1 mutations (Kato et al. 2000). Hyaline conglomerate inclusions have also been reported in ALS cases, however this type of inclusion body is not specific compared to ubiquitin inclusions (Corbo and Hays, 1992). Additionally, Bunina bodies, which are cystatin C and

tranferrin containing inclusions, are also found in motor neuron cell bodies and are present in 70% to 100% of ALS cases (Wijesekera and Leigh, 2009).

To date, there is no specific treatment available for ALS, however symptomatic and palliative treatments such as physiotherapy, ventilatory management and counselling have improved patients' quality of life (Wijesekera and Leigh, 2009). Riluzole, a glutamate antagonist is the only drug available that has been approved by the Food and Drug Administration as being safe and effective for treating ALS (Rowland and Shneider, 2001; Simmons, 2005). Riluzole is described as reducing the deterioration in muscle strength by suppressing the excitatory activity of glutamate receptors in the ALS pathogenesis pathway and has been reported to improve the survival rate by 12 to 18 months (Cheah et al. 2010). However, the effect of riluzole cannot be sustained after 18 months of treatment and stopping the medication at the advance stage of the disease should be considered (Traynor et al. 2003). Other glutamate antagonists such as the branched-chain amino acids lamotrigine and dextromethorphan were also investigated but had no beneficial effects in the clinical trials (Miller, 1999; Demaerschalk and Strong, 2000).

1.3.2 *SOD*1 Mutations in Familial ALS

The majority of familial ALS cases are inherited by an autosomal dominant pattern (Mulder et al. 1986) with a minority of cases inheriting through a recessive gene (Andersen et al. 1996; Yang et al. 2001). A major breakthrough in the understanding of familial ALS mechanism was made in 1993 and involved the discovery of the 11 pathogenic mutations in the *SOD*1 gene (Rosen et al. 1993). *SOD*1 is an antioxidant enzyme found mostly in the cytosol but also in the mitochondrial intermembrane space, nucleus and peroxisomes (Banci et al. 2008). The *SOD*1 gene is composed of five exons, which encode the 154 residue amino acid that is responsible for the catabolism of superoxide radicals to hydrogen peroxide and molecular oxygen (Bannister et al. 1991). The mature, correctly folded *SOD*1 is obtained through several post-translational modifications; copper and zinc ions binding, disulfide bond formation and dimerisation (Valentine et al. 2005). To date, more than 150 different *SOD*1 mutations have been reported (http://alsod.iop.kcl.ac.uk), with the majority being missense mutations (Ticozzi et al. 2011). These *SOD*1 mutations are distributed throughout the five exons, although larger numbers of mutations are found in exon four and five (Andersen, 2006; Ticozzi et al. 2011). The examples of missense mutations that produce distinct phenotypes are the A4V (alanine to valine at codon 4) and D90A (aspartic acid to alanine at codon 90)

(Pasinelli and Brown, 2006). The A4V is inherited through a dominant pattern and has been identified as the most common and aggressive form of the disease with a mean of survival of around one year (Deng et al. 1993). In contrast, the homozygous individuals of D90A have slower disease progression with a prolonged survival of more than a decade (see 1.3.3, page 46). With the exception of A4V, D90A and several other *SOD*1 mutations in familial ALS, the clinical features of other *SOD*1-linked ALS cases appear to be indistinguishable from ALS patients without a *SOD*1 mutation (Gros-Louis et al. 2006).

1.3.3 Recessive Inheritance of D90A *SOD*1 Mutation in ALS

Of the 150 *SOD*1 mutations that have been reported in familial ALS cases, only the D90A mutation has been associated with autosomal recessive inheritance, specifically in Scandinavia and Western European countries; however it has been shown to be dominantly inherited in other parts of the world (Andersen et al. 1995; Khoris et al. 2000; Jonsson et al. 2002). D90A *SOD*1 mutation has been reported to have a higher frequency in Scandinavia (1-2.5%) than elsewhere (<0.05%). A proportion of homozygous individuals who are symptom free have also been described (Andersen et al. 1995; Andersen et al. 1996). The phenotype-genotype relationship is further complicated by reports of heterozygous individuals with the D90A mutation, displaying a dominant trait with classic signs of ALS and survival between two to five years (Andersen et al. 2001). In addition, a more recent study has reported a compound heterozygote of D90A with a novel *SOD*1 mutation of D90N (aspartic acid to asparagine) (Hand et al. 2001). The authors in this study suggested that both mutations are required to develop the disease although speculation on D90N as a novel recessive mutation was proposed (Hand et al. 2001).

Cases with D90A mutation display a very characteristic and uniform disease phenotype compared to other patients with dominantly inherited *SOD*1 mutations (Andersen et al. 1996). The mean age of onset in homozygous D90A cases is 44 years, which is a decade earlier compared to classic ALS or sporadic ALS cases. There is no sex predilection detected in D90A patients. The onset of paraparesis is insidious and asymmetrical, and patients initially experience a pre-paretic phase with lower extremity stiffness, muscular cramps, clumsiness and general fatigue. Pain in the lumbar area, buttocks, hips and/or limbs have been reported during the early stage of the disease (Andersen et al. 1995). The period of the pre-paretic phase is highly variable between patients, ranging from a few months to several years during which time the clinical and neurological investigations are reported to be normal (Andersen et al. 1996). This phase slowly deteriorates to the paretic

phase with a combination of UMN and LMN systems of the lower limbs, generalised muscle atrophy, fasciculations, spastic muscle tone and increased spinal reflexes have been reported as common features (Weber et al. 2000). The disease gradually progresses to upper limbs usually affecting the UMN system before manifesting LMN signs. Upper extremity involvement appears on average 4.1 years after the initial onset (Andersen et al. 1996). The development of bulbar symptoms such as dysarthria and dysphagia is slightly varied between individuals with a mean of 5.4 years from the first disease onset (Andersen et al. 1996). Urgency of micturition and difficulty initiating urination are common in patients with advance stage of D90A mutation (Weber et al. 2000). Generalised muscle atrophy and tetraplegia may be observed before the patients die due to respiratory failure (Andersen et al. 1996). Inappropriate laughing and crying have been reported in some patients however no cognitive impairment has been observed. Specific pathological characteristics have not been reported in human patients with the D90A mutation however it is speculated to be similar to other *SOD*1 mutations (Andersen et al. 1996).

1.3.4 Proposed Underlying Mechanisms of *SOD*1 Mutations in ALS

The mechanisms involved in the selective motor neuron degeneration caused by *SOD*1 mutations in ALS remain unresolved, however, a plethora of hypotheses have been proposed (Ilieva et al. 2009; Rothstein, 2009). In this section, I summarise the current aspects of the pathogenesis of *SOD*1-linked ALS that may be particularly relevant to DM, including oxidative damage (Barber et al. 2006; Kabashi et al. 2007), protein misfolding and aggregation (Watanabe et al. 2001), mitochondrial dysfunction (Israelson et al. 2010) and non-cell autonomous motor neuron death.

The SOD1 enzymes are directly associated with the cellular antioxidant defence mechanism that are involved in catalysing the toxic superoxide radicals (Bannister et al. 1991). The global distribution of *SOD*1 mutations across all exons therefore intuitively suggests the loss of SOD1 function and hypothesises accumulation of free radicals and oxidative stress that eventually leads to motor neuron death in ALS (Deng et al. 1993). However, homozygote *SOD*1 knockout murine models reported in previous studies have failed to develop apparent motor neuron signs (Reaume et al. 1996; Ho et al. 1998), while transgenic murine models over-expressing mutant human *SOD*1 (G93A, G85R and H46R) do produce motor neuron degeneration and paralysis despite normal endogenous SOD1

activity (Gurney et al. 1994; Bruijn et al. 1997; Nagai et al. 2001). These observations lead to the proposition that motor neuron death in *SOD*1-linked ALS reflects acquired toxic properties of the mutant SOD1 protein rather than loss of function (Nagai et al. 2001; Rothstein, 2009). However, despite strong evidence of the gain of toxic SOD1 function in ALS pathogenesis, the hypothesis of the loss of function cannot be completely excluded (Turner and Talbot, 2008). More recent evidence has demonstrated that the *SOD*1 knockouts display multisystem abnormalities (Ho et al. 1998; Imamura et al. 2006; Elchuri et al. 2005) including significant locomotor deficits associated with peripheral axonopathy (Muller et al. 2006; Fischer and Glass, 2007). It remains unclear why *SOD*1 knockouts do not display distinctive motor neuron signs in earlier studies, however such a response is potentially caused by compensatory mechanisms that are yet to be discovered (Turner and Talbot, 2008).

The putative toxic gain of SOD1 protein mechanisms that induce motor neuron degeneration in ALS remains unknown, but may involve several complex interacting molecular pathways (Rothstein, 2009). The individual *SOD*1 mutations are scattered throughout the protein, which are predicted to interfere with different aspects of the protein structure depending on the location of the mutation (Valentine et al. 2005). This contributes to failure of the protein to fold properly leading to accumulation of misfolded SOD1 proteins and SOD1 aggregates or inclusion formation in motor neurons as observed in ALS patients (Bruijn et al. 1997; Watanabe et al. 2001). The accumulation of misfolded SOD1 protein subsequently activates the unfolded protein response (UPR), which is a quality control of cellular mechanisms that facilitate protein folding (Bento-Abreu et al. 2010). A potential cascade involves the accumulation of misfolded SOD1 within the ER, inducing ER stress. ER stress initiates the upregulation of a number of UPR enzymes and chaperones (e.g., PDI, BiP) as well as transcription factors (e.g., ATF6, XBPI) that alter protein translation rates (Atkin et al. 2006; Atkin et al. 2008). The clearance of misfolded SOD1 proteins can be mediated by the ubiquitin-proteosome pathway but there is evidence that this system may be disrupted in ALS (Urushitani et al. 2002). Collectively, these events may lead to motor neuron death.

Misfolded SOD1 proteins have been associated with mitochondrial perturbations by the aberrant deposition of the misfolded SOD1 proteins in the outer membrane of mitochondria (Vande et al. 2008). There is a clear implication that misfolded SOD1 proteins could bind directly to the voltage-dependent anion channel 1 protein (VDAC1)

(Israelson et al. 2010), which is embedded in the outer mitochondrial membrane that regulates metabolite exchange (eg., adenosine triphosphate and adenine nucleotides) and the release of reactive oxygen species (ROS) between mitochondria and cytosol (Han et al. 2003; Colombini, 2004). Therefore, the binding of misfolded SOD1-VDAC1 would disrupt the metabolite flux and the release of ROS from the mitochondria, leading to oxidative stress and mitochondrial dysfunction (Israelson et al. 2010). Such dysfunction can eventually induce morphological damage to mitochondria and activate apoptosis cascade events (Pedrini et al. 2010).

In addition to the potential mechanisms described above there is evidence to support a non-cell autonomous contribution to the viability of motor neurons in ALS (Ilieva et al. 2009). Transgenic mice expressing mutant *SOD*1 in motor neurons with wild type *SOD*1 in non-neuronal cells are not sufficient to induce ALS, which clearly implies that the non-neuronal cells may substantially contribute to the disease initiation (Clement et al. 2003; Yamanaka et al. 2008). The exact mechanism of a non-cell autonomous affect in ALS has not been fully delineated although a hypothesis has been proposed on the formation of misfolded SOD1 aggregates in the neighbouring glial cells; astrocytes and microglia that could subsequently trigger a series of neurotoxic factors including inflammatory cytokines and ROS, which potentially exacerbates the damage to the motor neurons (Harraz et al. 2008; Ilieva et al. 2009). The involvement of other non-neuronal cells such as Schwann cells (Lobsiger et al. 2009) and T-lymphocytes (Beers et al. 2008; Chiu et al. 2008) have also been implicated in ALS onset and progression.

The initial damage in ALS may take place within motor neurons however the involvement of non-neuronal cells may also directly contribute to the development of ALS pathology (Ilieva et al. 2009). Therefore, all proposed mechanisms, either loss or gain of function, are probably contributors to ALS pathogenesis through induction of damage within different cell types (Pasinelli and Brown, 2006; Turner and Talbot, 2008), although it remains to be established whether these mechanisms are involved in DM pathogenesis. The selective vulnerability of motor neurons in ALS with mutant *SOD*1 remains unexplained, although it may be related to the requirements needed to maintain long motor axons and the high energy demand of the cargo proteins involved in retro- and anterograde transport (Shaw and Eggett, 2000). Although the precise mechanisms remain unresolved, it is clear that motor neurons are very sensitive to oxidative stress and mitochondrial

dysfunction, and this may increase the vulnerability of these cells compared to others (Robberecht et al. 2000).

1.4 ALS is a Potential Orthologue of DM

The elucidation of disease mechanisms in ALS has relied heavily on transgenic animals expressing human *SOD*1 or other mutants to produce motor neuron disease that mimics many features of ALS (Bruijn et al. 1997; Deng et al. 2006; Jonsson et al. 2006). The use of transgenic animals in ALS research has provided significant insight into underlying disease mechanisms, while at the same time permitting the formulation of hypothesis testing and the safe evaluations of new therapeutic interventions prior to translation to human trials (Jonsson et al. 2006; Turner and Talbot, 2008). However, several limitations have been recognised in transgenic animal models. Transgenic animals are artificially produced, which often requires a high level of gene expression that may itself induce the pathological phenotype (Battistini et al. 2010). Although the clinical signs and pathological characteristics observed in these transgenic models may be similar to the human form of ALS, the findings often have limited relevance to human ALS because of profound differences in inter-species physiology (Boido et al. 2012). The primitive nervous system and limited cognitive capacity of transgenic models may not truly reflect the nervous system complexity as described in humans, even though they represent a very useful tool to investigate ALS (Boido et al. 2012).

The identification of E40K *Sod*1 mutation in DM has established a genetic link between DM and ALS, therefore implying DM as the first spontaneously occurring animal model of ALS (Awano et al. 2009). The clinical description of E40K *Sod*1 mutation is comparable to D90A *SOD*1 mutation (Vasquez, 2011). The pathologic features of DM that have been characterised to date to date are also comparable to those observed in ALS, including axonal degeneration with secondary demyelination and astrogliosis. Neurogenic muscle atrophy due peripheral nerve degeneration is also common to both DM and ALS. The cytoplasmic SOD1 aggregates and co-localisation of PDI have further highlighted the similarities of DM with ALS. In addition, the upregulation of UPR proteins; PDI, C/enhancer binding homologous protein (CHOP) and binding immunoglobulin protein (BiP or Grp78) were found to be significantly upregulated in DM spinal cords, indicating that ER stress is common to both ALS and DM (Long et al. 2012). This encouraging

progress in DM research has therefore strengthened the value of DM as an animal model of ALS.

The development of a large animal model based on a spontaneous SOD1 mutation may serve as an ideal alternative for investigating pathophysiology, development of diagnostic tools as well as therapeutic interventions in both forms of ALS. The dog is more similar to human in terms of the structure and complexity of the nervous system (Boido et al. 2012). The pattern of clinical progression in DM is relatively homogenous in comparison with the phenotypic heterogeneity in ALS. Such advantage may facilitate the evaluations of specific biomarkers and therapies for ALS that could be conducted in a clinical population in an environment that mimics human trials (Coates and Wininger, 2010). The DM-affected dogs with E40K mutation could be used to investigate a comparable *SOD*1 mutation in ALS (D90A), for instance, to identify modifying loci and environmental influences that may contribute to exacerbation or amelioration of the disease severity in both mutations (Awano et al. 2009). The common euthanasia of DM-affected dogs in early stage disease may also provide valuable tissue material that is rarely available from ALS patients. Based on these grounds, further characterisation of DM as a potential ALS model is critically required and can be accomplished through collaboration between research groups investigating ALS and DM.

The clinicopathological comparison between classic ALS, and DM is summarised in Table 1-2. D90A is potentially a closely related SOD1 mutation with that described in DM and therefore the characteristics are also included for comparison.

Disease Features	Classic ALS (spinal form)	Recessive D90A phenotype in familial ALS	DM
*SOD*1 Mutation	- 20% in familial ALS, 1-7% in sporadic ALS	- 1-2.5% in Scandinavia, less than 0.05% elsewhere	- 76-94% of DM-affected population harbouring E40K mutation
Mode of inheritance	- Mostly autosomal dominant pattern with few exceptions	- Recessive inheritance with incomplete penetrance - Heterozygous D90A appears as dominant trait	- Recessive inheritance with incomplete penetrance - The genotype-phenotype correlation of E40K heterozygote has not been fully elucidated
Signalment and disease progression			
The mean age of onset	- 60 years	- 44 years	- 9 years
The mean survival time	- 2-5 years	- 13 years	- 3 years (36 months)
Sex predilection (male:female)	- 3:2	- 1:1	- 1:1
Clinical progression	- Average 2.5 years from first onset to bulbar signs	- May vary from months to years - Average 4.1 years from first onset to upper limb involvement - Average 5.4 years post onset to bulbar symptoms	- 6-9 months from first onset to non-ambulatory paraparesis - LMN paraplegia within 9-18 months post onset - Thoracic limb involvement to brain stem signs stage within 14-36 months
Clinical signs			
Onset	- Insidious and asymmetrical, with first onset either in upper or lower limbs	- Insidious and asymmetrical with first onset in the lower limbs	- Insidious and asymmetrical, with first onset in pelvic limbs
First Symptoms	- Difficulty in lifting the upper or lower limbs - Stumbling or clumsiness - Muscle cramps and fasciculation are rarely noticed at this stage	- Muscle stiffness and cramps, clumsiness and general fatigue - Pain in lumbar or lower limb region	- Scuffing - Difficulty climbing/goind down the stairs

Table 1-2 : The clinic-pathological comparison between classic ALS, recessive D90A *SOD*1 mutation and DM.

Disease Features	Classic ALS (spinal form)	Recessive D90A phenotype in familial ALS	DM
Clinical signs (cont'd)			
Early stage	- Mixture of UMN and LMN signs although patients with UMN dominance have been identified - Asymmetric spastic paresis, muscle atrophy in upper or lower limb - Muscle cramps and fasciculation are more prominent	- Mixture of UMN and LMN signs - Asymmetrical, spastic paraparesis in lower limb - Muscle atrophy and fasciculations	- Initially start with UMN followed by LMN signs - Worn and bleeding claws due to scuffing - Asymmetrical spastic paraparesis with general proprioceptive ataxia in pelvic limbs - Crossing limb and swaying movement of pelvis - Mild muscle atrophy in pelvic limb
Late stage	- Bulbar signs; dysarthria followed by dysphagia - Facial weakness and tongue atrophy - Urgency in micturition/ incontinence and cognitive impairment are rare but have been reported	- Ascend to upper limbs - Development of bulbar signs - Urgency of micturition or difficulty in urination is common - Generalised muscle atrophy and tetraplegia are common at death - No cognitive abnormalities	- Ascends to thoracic limbs - Urinary and fecal incontinence are rare but have been reported - Bulbar signs; dysphagia and inability to bark - Generalised muscle atrophy and tetraplegia
Cause of death	- Respiratory failure due to respiratory muscle paralysis	- Respiratory failure	- Euthanasia - Natural cause is not determined but respiratory difficulty may be observed at late stage

Table 1-2 (cont'd): The clinic-pathological comparison between classic ALS, recessive D90A *SOD*1 mutation and DM.

Disease Features	Classic ALS (spinal form)	Recessive D90A phenotype in familial ALS	DM
Pathology			
Central nervous system	- Severe loss of Betz's cells and pyramidal neurons - Diffuse degeneration of spinal cord and secondary demyelination, specifically in the corticospinal tract in the lateral and anterior funiculi - Astrogliosis - Degeneration of neurons in cranial nerves nuclei V,VII,IX,X.XII in brainstem - Various inclusion bodies including SOD1 containing inclusions found in neurons and astrocytes	- Speculated to be similar with classic ALS - Lewy body-like hyaline inclusions or astrocytic hyaline inclusion containing SOD1 antigen	- Massive axonal degeneration with secondary demyelination at the lateral funiculus and dorsal column of the middle to caudal thoracic region - Astrogliosis - Mild gliosis and chromatolysis in grey matter - Neuronal loss, chromatolysis and gliosis particularly in red nucleus of the brain - SOD1 containing inclusions in motor neuron cell bodies of the spinal cord
Peripheral nervous system	- Axonal degeneration and demyelination of nerve fibres in ventral root particularly in cervical and lumbar region - Reduced number of neurons in dorsal root has been reported although sensory system is spared in most cases Neurogenic atrophic changes in mucles	- Speculated to be similar with classic ALS	- Nerve fibre loss, axonal degeneration and secondary demyelination in the dorsal root - Neurogenic atrophy changes in muscles

Table 1-2 (cont'd): The clinic-pathological comparison between classic ALS, recessive D90A *SOD*1 mutation and DM.

1.5 An Introduction to Protein Biomarker Discovery

1.5.1 General Concept

A biomarker or biological marker is a characteristic that can be measured objectively and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (Atkinson Junior et al. 2001). In a broad sense, the term "biomarker" is an index of a complex biological system that may be regarded as being cellular, biochemical, genetic, or a specific molecular alteration that gives rise to a parameter that can be measured in any biological media such as tissue, cells or body fluids (Garban et al. 2005). The concept of biomarkers have been applied in diagnostic medicine since 14th century or earlier, including the inspection of urine colour and sediment to detect urinary disease in human patients. Blood pressure, for instance is another example of an established biomarker, and has been used since 1901 to correlate elevated blood pressure and adverse cardiovascular outcomes (Desai et al. 2006). Since then the biomarker concept has evolved into a powerful approach that requires a combination of screening technologies that permits an understanding of underlying disease mechanisms at all levels.

The classic model of biomarkers is summarised in Table 1-3 (Sahu et al. 2011). However, the concept of biomarkers has evolved over time and has been defined from various viewpoints. A recent interpretation of the word biomarker is that biomarker in reality is an umbrella coalescence term that covers a vast number of disciplines, including the use and development of –omics tools and technologies, monitoring drug discovery and development processes leading to a more full understanding of the prediction, regression, outcome, diagnosis and treatment of disease. As the definitions suggest, biomarkers can be classified in many ways depending on their specific characteristics (eg., biochemical or physiological), technology used (eg., imaging, genomic or proteomic) and clinical applications (eg., diagnostic or therapeutic).

Type	Definition
Type 0	Natural history marker that correlates with known clinical indicators such as disease predisposition and severity
Type 1	Drug activity marker that reflects the response of therapeutic intervention, such as drug responses, optimization of doses regimes and monitoring of combination therapies.
Type 2	Surrogate marker that is intended to substitute for a clinical end point and expected to give a prediction on the clinical benefit

Table 1-3: The classic model of biomarkers.

Adapted by Sahu and Others (2011).

The ultimate goal of biomarker discovery is to develop screening tests for the early detection of diseases where patients can be advised and effectively treated in the early phase of the disease (Thatcher and Caputo, 2008). Therefore, the application of a biomarker is significantly relevant in chronic cases that may require extensive clinical investigation and complicated diagnosis (Tumani et al. 2008). The development of a biomarker requires extensive research including the often prolonged step of biomarker validation, with the aim to provide an understanding of the disease mechanisms that can be used and translated into clinical research. An ideal biomarker should be highly specific and reliable, and should be acquired with minimal intrusion and harm to the patient. It should also be sensitive enough to cope with many variables in general populations such as gender and ethnic group (Atkinson Junior et al. 2001). In addition, the clinical material for the ideal biomarker should be obtainable in a reproducible manner and the technology required for analysis must be easily accessible.

1.5.1.1 "Omics" Platforms for Biomarker Discovery

Technologies for high throughput scanning for biomarker discovery or the so called 'omics' revolution has evolved at a rapid pace, allowing systematic analysis of biomarkers in many diseases (Ghosh and Poisson, 2009). 'Omics' technology is characterised by a range of modern analytical instruments that have astonishing ability to identify and/or quantify biological molecules within a short period of time. Significant amounts of information from various biological media can be obtained, not just to improve diagnosis but also to provide a basis for understanding the mechanism of a number of physiological and pathological processes in a complex biological system (Casado-Vela et al. 2011). The global approach to 'omics' research that is being adopted in biomarker discovery can be

categorised into several subsets including genomics, proteomics, and metabolomics (Moore et al. 2007; Niedbala et al. 2009). It is anticipated that the use of these applications would escalate biomarker detection by allowing scientists to overcoming some important obstacles in biomarker research (Rolland et al. 2012; Truong et al. 2012). However the complexity of biological systems precludes progress in biomarker discovery and data generated by investigating individual components in isolation may be difficult to interpret. Therefore, in the past few years, biomarker-based research is geared towards an integrative approach of 'omics', and has been a preferable approach to generate more specific and sensitive biomarkers for diseases (Aggarwal and Lee, 2003; Robeson et al. 2008).

Genomics is the discipline of studying genomes in organisms, and concerns the structure, function, evolution and mapping of genomes (Fertig et al. 2012). The application of genomics in biomarker discovery has made substantial progress towards understanding the genetic linkage (such as mutations and polymorphisms) in diseases such as cancers (Garman et al. 2007; Dallol et al. 2012) and neurodegenerative disorders (Borovecki et al. 2005; Weinberg and Wood, 2009), and has facilitated the development of specific diagnostics and therapeutics based on the genetic variations and disease predispositions. The technology of genomics spans a variety of methods used to investigate gene expression, transcript level profiling, gene sequencing, and DNA microarrays (Wilson et al. 2004; Niedbala et al. 2009). Emerging themes in genomic technologies also include whole genome sequencing, microRNA and epigenetics (Casado-Vela et al. 2011). Although genomics are able to provide significant amounts of information on gene structure and activity, the behaviour of gene products are difficult to predict, due to complex gene regulation processes at the level of translation (Dove, 1999). Unlike the relatively unchanging genome, the dynamic proteins in any particular cell change dramatically in response to the biological events such as post-translational modifications, aging, stress, as well as drug or pathologic responses (Cho, 2007). Thus, genomics information in isolation does not provide a complete profile of protein abundance and its structure and function.

Proteomics is a field that promises to bridge the gap between genes and cellular activities, that has the capability to comprehensively examine protein expression, structural variation and protein-protein interaction (Wilkins et al. 1996). Proteomics approach has been applied in various areas of medicine, ranging from deciphering molecular pathogenesis of

disease to the identification of novel drug targets and the discovery of potential diagnostic biomarkers (Frank and Hargreaves, 2003; de Vera et al. 2006). The emergence of proteomic technologies is being driven by the development and integration of automated large-scale analytical instruments at the same time as the emergence of sophisticated bioinformatics approaches for analysing convoluted proteomics data (Tyers and Mann, 2003; de Vera et al. 2006). Technologies employed in proteomic based investigations include the combination of protein separation tools and protein identification by high resolution mass spectrometry (see 1.5.3, page 61) (Aebersold and Mann, 2003; Cho, 2007).

Another rapidly emerging 'omics' technology is metabolomics (Patti et al. 2012). Metabolomics involves the analysis of the profile of metabolites from a repertoire of cells (Frezza et al. 2011), specific tissues (Li et al. 2011), organs (Wishart, 2006) and biological fluids (Gieger et al. 2008; Suhre et al. 2010). The identities, concentrations and changes within these compounds result from complex interactions between gene and protein expression as well as the environment, and this information when collected in an integrative and comparative manner with genomics or proteomics is potentially useful. (Kaddurah-Daouk et al. 2008). Several platforms of metabolomic technology have been described in the literature and include nuclear magnetic resonance (NMR) spectroscopy, high-performance liquid chromatography and mass spectrometry based platforms (Kaddurah-Daouk et al. 2008; Patti et al. 2012). Furthermore, the high-throughput nature of metabolomics is particularly ideal in performing biomarker screening for diseases or for following drug efficacy and increases the ability to predict individual variation in drug response phenotypes (Coen et al. 2004; Lindon et al. 2004).

1.5.1.2 Challenges and Limitations

Despite high throughput technologies in developing biomarkers, the characterisation of a clinically useful biomarker is not straightforward and often requires an extensive period of research from initial discovery to subsequent validation (Niedbala et al. 2009). The restricted sourcing of clinical material due to ethical restrictions alongside quality of sample obtained are considered as central issues that cannot be overlooked and will ultimately affect the quality of biomarkers produced. This affirms the need for collaboration and continuous interaction between researcher and clinician. The biological stability of substances is also critical and requires thorough assessment if long-term storage is needed (Ferguson et al. 2007). Consistency when handling and processing the samples may therefore alleviate this problem (Pieragostinoa et al. 2010).

The validation process for biomarkers requires confirmation of precision in terms of the assay's efficacy and reproducibility. Most importantly, the association of the biomarkers with clinical and pathological features of the specific diseases must be established. These requirements are complicated by the diversity and inherent inconsistencies in biological systems and by the differences between individuals, caused by the limitless variables from genetic background, environment, ethnic groups, diet, age and gender (Mayeux, 2004). These variables can create background 'noise' in biomarker identification and a failure in considering these factors may influence the validity of clinical studies. Fortunately, these variables can be selectively controlled in research and clinical trials, although the outcomes may not necessarily represent the global disease population.

Reliability and reproducibility in biomarker investigations is also crucial in order to facilitate the validation process (Dougherty, 2012). A reliable biomarker must be capable of being reproducibly quantified in the independent laboratory by independent personnel. The lack of reproducibility has become one of the common problems in biomarker validation which can be contributed to lack of standardisation in multi-step protocols between personnel or laboratories (Silberring and Ciborowski, 2010) as well as equipment errors (de Vera et al. 2006). A well-organised manual for procedures outlining the details from sample collection to analysis and continuous interaction between laboratories may alleviate this problem. Concerns of the availability of samples and their replicates that accurately reflect the diseased and non-diseased groups have also been reported to affect the reproducibility of biomarker identification (Issaq et al. 2011). Although it is ideal to generate a large sample number for biomarker analysis driven by statistical power analysis, the predicted sample size is not always practical in a clinical environment.

The biomarker discovery in medicine clearly exerts an enormous potential, however without proper planning of experimental design and sample management, the efforts and expectations may very easily hampered. The specific limitations described in this section are often underestimated and as a result, many potential biomarkers may not be validated and fail to reach the desired endpoint.

1.5.2 Biological Fluids Vs. Tissue Material

An important consideration in protein biomarker investigation includes the selection of an appropriate type of sample as well as the practicality of collecting the sample of interest (Muschik et al. 2008). Besides using the 'gold standard' samples obtained from a variety

of biological media including tissues or biological fluids, other factors such as having control material that is significantly different in respect of the disease of interest including an age-matched control group should be considered, although sometimes this is not achievable (Rifai et al. 2006). Investigative studies using tissues obtained from biopsy or post mortem afford the best opportunity of discovering novel biomarkers as it is not subjected to the dilution effect imposed on biological fluids, which may therefore require highly sensitive detection methods (Fang et al. 2009). However, selecting appropriate tissue samples for biomarker identification can be challenging as the distribution of the non- and diseased cells are typically heterogeneous and may complicate the data interpretation (Lahdesmaki et al. 2005). Fresh tissue specimens may not always be available, possibly due to the invasive nature of biopsy techniques, particularly involving CNS material (Dunckley et al. 2005). Tissue collection from post mortem is more practical however the variability of the patient's agonal status may influence the specific biomarker parameters and reduced the sensitivity of biomarker detection (Perry et al. 1982; Harish et al. 2011). This material also represents the end stage of the disease and therefore the proteome profiles could be further impacted by secondary pathogenic events. Furthermore, the establishment of a large enough archive of tissue may take years to accomplish (Dunckley et al. 2005).

In general in the last decade biological fluids have garnered more attention in protein biomarker research due to their easy accessibility and availability compared to tissue material (Alrawashdeh and Crnogorac-Jurcevic, 2011). Biological fluids are dynamic components that largely reflect the physiological and pathological changes in the organ or tissues they come in contact with, and therefore may represent a rich source for biomarker discovery (Rifai et al. 2006; Alrawashdeh and Crnogorac-Jurcevic, 2011). Blood (serum and plasma) has been a common source and the most studied in protein biomarker discovery (Anderson and Anderson, 2002; Good et al. 2007), however other body fluids such as CSF (Kroksveen et al. 2010), urine (Wu et al. 2010; Coca and Parikh, 2008), saliva (Chiappelli et al. 2006; Kinney et al. 2011), ascitic fluid (Gortzak-Uzan et al. 2008; Kashyap et al. 2010), bile and gastric juices (Deng et al. 2011), and tears (Zhou et al. 2009) have also been explored. The use of biological fluids in protein biomarker research also has a great potential in large scale investigation for developing diagnostic assays (Good et al. 2007). The method of collection is always low cost, either for single or multiple samples, at the same time as avoiding the risk of performing invasive tissue

biopsies in patients, although this is not entirely true for certain biological fluids such as bile and gastric juices (Alrawashdeh and Crnogorac-Jurcevic, 2011).

Biomarker investigations in biological fluids come with their own set of challenges. The level of complexity of biological fluids is a significant limitation in biomarker discovery, since these biological media may consist of a complicated array of molecules such as lipids, proteins, and carbohydrate compound that may interfere or mask the analysis of molecules that are present in low concentration or near the limit of detection (de Vera et al. 2006; Good et al. 2007; Thatcher and Caputo, 2008). Major abundant proteins such as albumin and immunoglobulin have also been described as 'biological noise' that can mask the low abundance molecules in biological fluids (Good et al. 2007). The depletion of these abundant proteins may improve the biomarker detection, however may also cause significant loss of proteins (Mayeux, 2004; De et al. 2010). The other limitation when dealing with biological fluids involves the pre-analytical variables, which can occur at any point from sample collection to the actual sample analysis (Ferguson et al. 2007). Therefore, regardless of the type of biological fluids employed, careful strategies on biomarker approach and sample management should be thoroughly assessed to improve the yield of quality biomarker.

1.5.3 Proteomic Technologies

The initial definition of 'proteome' analysis is the study of the entire protein complement expressed by a genome or by a cell or tissue type (Wasinger et al. 1995). Proteomics complements the study of genomes and transcript data, reflecting the true biochemical outcome of genetic information. Over the years, proteomics has evolved and become an advanced discipline that demands extensive investigations of proteins, from identification, quantity or abundance, posttranslational modification, binding molecules, and intracellular stability of proteins in complex biological systems (Doherty and Beynon, 2006). The proteomic approach has been widely used to identify biomarkers and understand the underlying disease mechanisms (Anderson and Anderson, 2002; Drabik et al. 2007; Issaq et al. 2011). Biological fluids have been preferably employed in protein biomarker research, with blood as a universal source for biomarkers, while the utility of CSF or urine may be restricted to the specific type of disease (Rifai et al. 2006).

Classical proteomic work involves a protein separation step, which can be categorised as gel- and non-gel-based techniques (Westermeier and Marouga, 2005). Each of these

methods offers unique advantages but also suffers from substantial drawbacks. Therefore, the selection of the appropriate method is highly important prior to sample employment. The protein separation step is usually coupled with advanced mass spectrometry (MS) techniques, which has been a common denominator in proteomics that enables accurate protein identification in a given sample (Aebersold and Mann, 2003). In this section, we present a brief description of the basic proteomic technologies that are currently being used in biomarker projects.

1.5.3.1 Protein Separation Techniques

1.5.3.1.1 Gel-based Techniques

The gel-based methods comprise one-dimensional electrophoresis (1-DGE) and two-dimensional electrophoresis (2-DGE). The application of 1-DGE technique may be conventional, but highly valued and have been used for at least 40 years to fractionate or separate the protein components of a sample (Oledzka et al. 2012). The technique is simple, and does not require complex sample preparation. The 1-DGE provides direct comparison between protein profiles and can be subsequently stained with commercially available Coomassie blue and silver staining reagents that are compatible with advanced MS methods. However, the protein profiles in 1-DGE are only marginally quantifiable and separation based on protein molecular weight is limited to those proteins between 10 and 250 kiloDalton (kDa).

Two-DGE offers more specific protein separation and is a commonly employed technique, since the core equipment is not prohibitively expensive (compared to the non-gel-based technique) and does not require dedicated specialists to utilise the equipment. This method first separates proteins by isoelectric focusing, which is based on their net charge and is followed by separation on a second dimension on polyacrylamide gel, which separates the proteins based on their size (Monteoliva and Albar, 2004). The combination of size and charge are often unique to a particular protein and the ‘spots’ generated can be subsequently identified by MS. One of the biggest limitations of this technology is the reproducibility of the profiles generated by 2-DGE although this has been partially overcome with the availability of affordable precast gels and reagents. Further development of 2-DGE also includes difference gel electrophoresis (DiGE) that allows for pre-labelling of the proteins with spectrally distinct dyes. The mixed samples can then be analysed on the same gel and the degree of overlap or non-overlap of the protein spots can be assessed by scanning the gel at distinct wavelengths. This technique minimises the time

involved to conduct the analysis and may improve the quality of the result by minimising the risk of inter-gel variation compared to conventional 2-DGE (Kolkman et al. 2005; Westermeier et al. 2008a).

1.5.3.1.2 Non-gel-based Techniques

The non-gel based strategy involves coupling a pre-fractionation step to direct MS analysis. The techniques include liquid chromatography (LC) and capillary electrophoresis (CE) separation techniques (Monteoliva and Albar, 2004) that allow for pre-fractionation of the source material to generate a more manageable sample and allow optimal resolution by MS. LC fractionates proteins according to their specific properties, either protein charge by ion exchange chromatography (Makawita et al. 2011), size of protein using gel filtration technique (Tantipaiboonwong et al. 2005), hydrophobicity through reverse phase chromatography (Alley, Jr. et al. 2010) or binding of specific ligands, such as antibodies, using affinity chromatography (Yang et al. 2006). These pre-fractionation techniques are then capable of generating a refined protein population that can then be analysed by MS. However as in the 2-DGE method, LC also suffers from the issue of reproducibility (Washburn et al. 2003). Recent developments of two-dimensional liquid chromatography have recently been adopted in the separation of complex mixtures in diverse fields, where the protein fractionation is performed by a combination of two technologies such as ion exchange and reverse phase chromatography (so-called MudPIT) (Westermeier et al. 2008b; Francois et al. 2009). With this set-up, more specific proteins can be identified in a fully automated manner with minimal handling of the sample (Tian et al. 2010).

CE is another emerging technology that offers several advantages including fast separation and high resolution, enabling robust detection of potential biomarkers (Kolch et al. 2005). The CE separations are facilitated by the use of high voltages (10-30 kV), which may generate electro-osmotic and electrophoretic flow of buffer solutions and ionic species, respectively, within the capillary (Huck et al. 2012). CE has been shown to be a powerful separation method for intact proteins with a high efficiency in the identification of large proteins compared to conventional LC (Mischak et al. 2009; Desiderio et al. 2010). Over the years, various interfaces with MS technologies have been developed (Klampfl, 2006), which have enhanced the utility of CE. The advancement of sample preparation methods has also reduced the length of time taken and increased the sensitivity of this technique. The application of CE in biomarker discovery has increased during the last five years (Klampfl, 2006), resulting in a significant number of proteins being identified in a range of

biological fluids, particularly in urine (Zurbig et al. 2009; Mischak et al. 2010) and CSF (Wittke et al. 2005; Jahn et al. 2011).

1.5.3.2 Protein Identification by Mass Spectrometry

Analysis by MS is central to proteomic studies (Rifai et al. 2006; Yates et al. 2009). The development in MS technology and accompanying software has greatly enhanced the speed of analysis and data interpretation (Westermeier et al. 2008c). All MS technologies, regardless of type, ionisation source, performance characteristics, operate according to the same basic principle, which produces a mass spectra with an output plot of the mass-to-charge (*m/z*) ratio of ions based upon their motion in an electric or magnetic field (Cho, 2007; Yates et al. 2009). MS comprises three essential components, an ionisation source, a separation manifold and a detection system. In general, the molecules in the source material (proteins, peptides, metabolites) are ionised in the gas phase, subsequently separated according to their *m/z* ratio, and propelled towards the analyser by virtue of charge repulsion. The spectra recorded by the detector are stored using appropriate software and the identification of proteins is performed by an interrogation of search engines utilising available databases. A more detailed description on MS technologies is beyond the scope of this thesis however Table 1-4 provides a list of various ionisation sources in MS systems together with the types of mass analysers that are compatible with each system.

Ionisation Source	**Mass Analysers**
Matrix assisted laser desorption/ionisation (MALDI) and Electrospray ionisation (ESI)	Time-of-flight (TOF)\ Quadrupole Ion trap Orbitrap™ Fourier transform ion cyclotron (FT-ICR)
Surface-enhanced laser desorption/ ionisation (SELDI)	TOF

Table 1-4: Type of ionisation sources and mass analysers in MS.

MALDI coupled with TOF analyser has been a common analytical method for protein detection (Benagli et al. 2011). MALDI involves ionisation of protein compounds that have been incorporated in crystalline structure or matrix, which are then irradiated by laser energy at a certain wavelength (Westermeier et al. 2008c). The rapid laser heating causes

desorption and ionisation of the analytes into the gas phase that are subsequently accelerated to the analyser (Yates et al. 2009). The mass determination is based on the application of constant voltage and the velocity of an ion when it reaches the detector (Reinhardt and Lippolis, 2011). This instrument has a number of advantages including a relatively straightforward procedure while at the same time providing high sensitivity and resolution with a small sample volume (Reinhardt and Lippolis, 2011). However, the reproducibility of this system is strongly dependent on the sample preparation as it is highly sensitive to contaminants such as salts (Yates et al. 2009; Dubois et al. 2012).

1.6 Cerebrospinal Fluid Protein Biomarkers:Advantages and Practical Considerations

1.6.1 CSF as an Ideal Biomarker Source for Chronic Neurodegenerative Disorders

CSF is a clear, colourless fluid that is predominantly produced by choroid plexus (Redzic et al. 2005; Sakka et al. 2011). CSF circulates within the ventricles of the brain and subarachnoid space of the CNS system (Martins et al. 1972). For many decades, the primary function of CSF has been described as a "protective buoyancy jacket" of the CNS (Redzic et al. 2005), however there is more recent evidence of its specific role in modulating intracranial pressure and excretion of toxic by-products produced from brain metabolic processes (Di Terlizzi and Platt, 2006). In addition, CSF contains various proteins, electrolytes, enzymes, neuropeptides and other biochemical compounds and also has a filtration function allowing movement of water-soluble substances from brain parenchyma into the CSF (Di Terlizzi and Platt, 2006; Johanson et al. 2008).CSF contains high salt with a very low protein concentration, that is approximately 200 fold lower than in plasma (Ramstrom and Bergquist, 2007). In dogs and cats, the CSF protein concentration is approximately 500 fold lower than in plasma (10-40mg/dl compared to 5-7g/dl) (Di Terlizzi and Platt, 2006). The main fraction of protein in CSF originates from blood, (eg., albumin) which constitutes 35-80% of the total protein in CSF (Reiber and Peter, 2001). Only 20% of CSF proteins are predominantly brain-derived (Reiber and Peter, 2001).

CSF has been considered a promising source of biomarkers particularly for chronic neurodegenerative disorders (Tumani et al. 2008). The alteration of protein levels, post-

translational modifications or turnover within the CSF may be reflected by different physiological and pathological conditions (Di Terlizzi and Platt, 2006; Tumani et al. 2008). CSF proteins are also more likely to be unique and potentially CNS-specific, therefore fit the criteria of an ideal biomarker of chronic neurodegenerative disorders (Tumani et al. 2008). Over the past few years, several groups have characterised the human CSF proteome to establish CSF biomarkers for major human neurodegenerative diseases including Alzheimer's disease (AD) (Finehout et al. 2007) and Parkinson's disease (PD) (Van Dijk et al. 2010) and ALS (Ranganathan et al. 2005; Pasinetti et al. 2006). Together, these studies have generated over 1500 CSF candidate proteins that are altered in CSF, although very few candidate proteins have been successfully translated into clinical practice (Abdi et al. 2006). An example of CSF biomarkers that have been well-established and validated include amyloid beta1-42 (Aβ1-42), total-tau (t-tau) and phosphorylated-tau-181 (p-tau-181) proteins in AD (Humpel, 2011). The combination of these CSF biomarkers with supportive clinical findings has significantly increased the diagnosis of sporadic AD, which yields a combined sensitivity of 95% and a specificity of 85% (Blennow, 2004; Marksteiner et al. 2007; Blennow et al. 2010). However the successful use of protein-based biomarkers for ALS has yet to be accomplished, although several candidate proteins have been identified as promising CSF biomarkers for ALS, which are further described in 1.7, page 68.

1.6.2 Practical Considerations Pertaining to CSF Proteomics

The sampling of CSF represents the most direct and convenient means to study the biochemical changes occurring in the CNS. However, the complexity and dynamic range of protein concentrations and protein heterogeneity in the CSF and the complexity of CSF as a biological fluid does create significant challenges to the existing proteomic technologies (Yuan and Desiderio, 2005). The combination of proper sample management, pre-analytical considerations as well as application of highly sensitive proteomics technology may facilitate the progress of the protein-based biomarker study towards success (Ferguson et al. 2007; Kroksveen et al. 2010).

1.6.2.1 Common Issues Pertaining CSF

Although CSF may be a valuable repository for potential biomarkers of CNS disorders, the availability of the relevant clinical material is limited and determined by clinical requirements. CSF sampling requires an invasive method of collection particularly in

lumbar collection compared to blood, saliva or urine samples (Di Terlizzi and Platt, 2009; Alrawashdeh and Crnogorac-Jurcevic, 2011). The CSF composition may be influenced by various biological variables including fluctuation of plasma composition, pathogenic processes, age and medications that can potentially give rise to an atypical biomarker signature (Zhang et al. 2005). Obtaining a sufficient volume of CSF (recommended volume between 1-2ml) for biomarker investigations is imperative (Teunissen et al. 2011), but is not always feasible, particularly from companion animals within the clinic environment. CSF has a very low protein concentration therefore a large volume of CSF may be required for biomarker analysis to achieve the minimum threshold of detection. In addition, CSF is easily contaminated by peripheral blood during sample procurement, which is more common in CSF lumbar collections. The iatrogenic blood contamination could dramatically alter the CSF protein profile and stability, subsequently confounding the CSF biomarker identification (You et al. 2005; Teunissen et al. 2009).

CSF is known to have a high salt content (>150mmol/l) (Yuan and Desiderio, 2005). The high concentration of salt has the potential to reduce efficiency of protein separation in gel electrophoresis, as it carries endogenous charge, which then affects the protein migration in the ampholyte matrix (Yuan et al. 2002). The high content of salt may affect the ionisation in mass spectrometry, suppressing the peptides and protein signal (Drabik et al. 2007). The potential biomarker candidates in CSF may be secreted or recruited from CNS by the blood-brain barrier, and is likely to be present at very low concentrations (Wetterhall et al. 2010). The detection of the low abundance proteins could be masked by more abundant proteins in CSF such as albumin (50-70%) and immunoglobulin (5-12%) (Reiber and Peter, 2001; Di Terlizzi and Platt, 2006). The employment of desalting and abundant protein depletion steps in CSF preparation protocols prior to proteomics has been proven to improve biomarker detection (Carrette et al. 2003; Khwaja et al. 2006). However, despite a vast range of commercially available kits for desalting and protein depletion available for CSF, poor protein recovery and variablity of efficacy of these preparation protocols remains a critical issue in CSF proteomics.

1.6.2.2 Pre-analytical Variables in CSF

In order to obtain reliable results and reduce the risk of detecting false biomarker candidates, proper sample handling is important to minimise the effect of pre-analytical variables on specific CSF proteins (Rosenling et al. 2009). The clinical or laboratory environment can be a potential source of limitless variables affecting the CSF proteome,

which may occur at any point from sample collection and laboratory storage, thus enhancing the risk of introducing poor quality samples into proteomic biomarker investigation (Ferguson et al. 2007). The net effect may introduce significant alterations on many CSF proteins and compromise data interpretation (Ranganathan et al. 2006). Therefore, identification and assessment of potential pre-analytical variables is imperative prior to CSF biomarker investigation and may accelerate biomarker detection and translation into clinical practice.

CSF in companion animals can be obtained either from cisterna magna or lumbar cistern (Di Terlizzi and Platt, 2009). Therefore, there is potentially a gradient effect with the composition of CSF proteins influenced by the site of sampling, although this has not yet been systematically investigated (Ferguson et al. 2007). The impact of blood contamination of CSF may alter the CSF protein stability due to the presence of blood-borne proteases (You et al. 2005). One of the most significant pre-analytical variables that can arise from clinical environment includes CSF handling and processing temperatures (Ferguson et al. 2007). CSF samples may be collected during non-operational hours and often temporarily stored either at room temperature or at 4°C for various lengths of time before being transferred into long-term storage (Bienzle et al. 2000; Fry et al. 2006). This handling practice may be ideal to preserve cellular morphology of CSF, however could potentially destabilize and alter the protein concentrations (Fry et al. 2006; Kaiser et al. 2007). Frequent freeze-thawing of CSF samples could also cause protein aggregation and has been found to exert marked effects on protein profiles, and therefore should be avoided whenever possible (Rosenling et al. 2009). Many clinical proteomic studies are performed using CSF samples that have been stored over timescales varying from weeks to several years (Teunissen et al. 2009). Although it is a now a consensus that -80ºC storage is optimal for biological samples such as CSF, for the past several years -20ºC storage had been considered practical and economical. Studies have shown that the prolonged storage of CSF in -20ºC could result in a significant reduction of protein concentrations as well as abnormal protein cleavage or truncation (Carrette et al. 2005; Boccio et al. 2006).

1.7 The Development of CSF Protein Biomarkers in ALS

The current diagnostic criteria of ALS is solely based on clinical assessment and genetic testing (see 1.3.1, page 42), therefore, given the lack of diagnostic tests and limited understanding of ALS pathogenesis, many opportunities exist for developing protein

biomarkers for ALS. As a result, tremendous efforts have been made in the identification of CSF biomarkers in ALS, although many of them are yet to be validated for clinical trials (Ryberg and Bowser, 2008). The biomarker discovery in ALS aims to improve early disease detection since the majority of ALS cases are diagnosed on average a year after first onset (Leigh, 2007). In addition, the successful characterisation of ALS biomarkers may lead to early intervention with riluzole, which can prolong patients' survival, as well as assisting in the identification of new therapeutic targets (see 1.3.1, page 45).

Biomarker discovery in ALS is universal in nature and rarely targets specific ALS forms or phenotypes. It is plausible that the extreme heterogeneity of ALS in the context of aetiology (genetic and potentially environmental), disease onset, progression and molecular pathogenesis could confound the ability of clinicians and researchers to identify homogeneous ALS subgroups for biomarker studies (Ganesalingam and Bowser, 2010). The heterogeneity issue also emphasises the need for the identification of multiple biomarkers to facilitate diagnosis and monitor ALS progression (Ryberg and Bowser, 2008).

In this section, I aim to discuss the development of CSF biomarkers in ALS by proteomics, with emphasis on selective CSF proteins that have been described as potential candidates in ALS literature. To date, over 40 different CSF proteins have been investigated in ALS (Ryberg and Bowser, 2008). One of the large groups of proteins is hormone and growth factors, particularly hepatocyte growth factor (HGF) and a hormone regulating blood cell production, erythropoetin (EPO). HGF has been shown to be upregulated in two independent studies (Kern et al. 2001; Tsuboi et al. 2002), although it has also has been reported in other neurodegenerative diseases such as AD (Tsuboi et al. 2003) and PD (Salehi and Rajaei, 2010). The EPO levels, in CSF from ALS patients, has been reported to be significantly reduced, a situation that is recognised as unique for ALS (Brettschneider et al. 2006b; Brettschneider et al. 2007). Other proteins belonging to this group that have shown altered levels include FGF-2 (Johansson et al. 2003), GNDF (Grundstrom et al. 2000) and PEDF (Kuncl et al. 2002).

The second group of proteins involves interleukins and immune-related proteins, which include RANTES (regulated on activation, normal T cell expressed and secreted), Flt3 (FMS-like tyrosine kinase 3) (Ilzecka, 2006) and MCP-1 (monocyte chemoattractant protein-1) proteins. These proteins have been reported to be upregulated in ALS patients' CSF, however only MCP-1 protein has been considered a promising candidate for ALS

since the levels are consistently increased in ALS CSF between studies (Wilms et al. 2003; Baron et al. 2005; Nagata et al. 2007).

Several neuron-specific proteins, including tau protein, (Jimenez-Jimenez et al. 2005; Brettschneider et al. 2007), p-tau (Sjogren et al. 2002) and neurofilament (heavy and light subunits) (Norgren et al. 2003; Brettschneider et al. 2006a; Zetterberg et al. 2007) have been investigated in ALS CSF. Both neurofilament heavy and light subunits are reported to be significantly upregulated in several studies (Rosengren et al. 1996; Norgren et al. 2003; Brettschneider et al. 2006a; Zetterberg et al. 2007). Although neurofilament proteins are not specific to ALS, the high level of these proteins is associated with rapid progression of ALS and may serve as a diagnostic and prognostic biomarker for ALS (Brettschneider et al. 2006a; Zetterberg et al. 2007). The assessment of t-tau and p-tau proteins in ALS patients' CSF revealed conflicting results, with most studies reporting the levels being unchanged (Sjogren et al. 2002; Brettschneider et al. 2007).

Biomarker evaluations on enzymes and enzyme inhibitors have been reported in ALS CSF. Cystatin C is down-regulated in ALS CSF (Tsuji-Akimoto et al. 2009) while in one study the assessment of SOD1 protein levels demonstrated a significant increase (Jacobsson et al. 2001; Frutiger et al. 2008) but not in other investigations (Zetterstrom et al. 2011). Other proteins, such as TIMP metallopeptidase inhibitor-2 (TIMP-2), matrix metalloproteinase-2 (MMP-2) (Lorenzl et al. 2003) and matrix metalloproteinase-9 (MMP-9) (Beuche et al. 2000) are unaltered whereas TIMP metallopeptidase inhibitor-1 (TIMP-1) levels have shown conflicting result in different studies (Beuche et al. 2000; Lorenzl et al. 2003)

A recent proteomic study of human CSF from ALS patients have characterised a panel of CSF biomarkers, which include cystatin C, transthyretin (TTR), and neuroendocrine protein 7B2 (Ranganathan et al. 2005). In another study, a neurosecretory protein VGF is also identified together with cystatin C (Pasinetti et al. 2006). The neuroendocrine 7B2 is reported to be elevated, while TTR and VGF are significantly reduced in ALS CSF. The observations of TTR and cystatin C reductions in CSF are consistent with several ALS studies, therefore have been described as highly potential candidates for ALS biomarkers (Ranganathan et al. 2005; Wilson, 2011).

1.8 The Clinical Impetus for the Development of a Biomarker in DM

A definitive diagnosis of DM is complicated by the lack of diagnostic tests in the clinic, with patient management relying on the "best estimation" and the necessity for post mortem examination for confirmation (Coates and Wininger, 2010). There is also a lack of disease markers that can be used to objectively understand the progression of the disease which negatively impacts on clinical decision-making and development of treatment regimes. The establishment of a *Sod*1 mutation in DM has made significant advancement in delineating a genetic basis in DM at the same time as implying DM as a potential orthologue of ALS (see 1.2, page 40). In addition, the *Sod*1 mutation has been widely used as a genetic marker for DM, which has significantly improve DM prediction in the clinic (Awano et al. 2009). However, genetic screening against 118G>A *Sod*1 mutation in DM does not provide confirmative diagnosis in the clinic therefore there is a clinical need for the establishment of biomarkers for DM that can be used as a complementary, specific diagnostic test to support the diagnosis of DM.

The extensive research and emerging publications on ALS have provided interesting insights and hypotheses that could be adopted in DM research (Coates and Wininger, 2010). Biomarker studies in ALS have listed promising biomarker candidates that could be substantiated in the DM model. Therefore, the evaluation of relevant ALS biomarkers in canine DM is pertinent and may have a major impact in the diagnosis and future development of therapeutic targets in DM. The recent advancement in and increased availability of proteomic technologies could also facilitate the biomarker development in DM, offering a great promise of understanding the disease mechanism in DM. Based on a significant understanding of the genetic basis in DM and the accessibility of biomarker technology with an established collaboration between clinician and researchers, this objective has become a real possibility.

1.9 Hypothesis and Aims of Research

The main aim of this research is to establish potential CSF biomarkers for DM. We hypothesised that canine CSF is an appropriate biological material to identify potential protein biomarkers for DM that can be used to define the diseased population and increase confidence in clinical diagnosis when used in combination with *Sod*1 genotyping. The

*Sod*1 genotyping against 118G>A would be developed in this project and used as a supportive diagnostic tool for the selection of DM cases. The biomarker discovery in DM was approached initially by evaluating the utility of the selected ALS biomarkers in DM CSF, followed by the application of proteomic techniques to survey for other potential markers. In addition, the assessment of the potential pre-analytical factors that may arise in this project was also addressed.

The background to each part of the project, specific aims and relevant studies are discussed in greater detail within each chapter.

2 General Materials and Methods

2.1 Overview

The research design and methodologies are summarised in Figure 2-1. The clinical samples; CSF, blood in EDTA (ethylenediamine-tetraacetic acid) and plasma were collected in University of Glasgow Small Animal Hospital (UGSAH). Genomic DNA was extracted from EDTA blood and subsequently utilised for genotyping against 118G>A *Sod*1 mutation. CSF samples were utilised for biomarker investigation, which was conducted in two phases; 1) characterisation of selected ALS biomarkers in DM CSF by Western blot technique and 2) identification of novel CSF biomarkers in DM CSF by 1-DGE and MALDI-TOF MS. Potential candidate proteins that were successfully demonstrated in DM CSF were used for selected stability characteristics, where the effects of CSF sample handlings were evaluated. The validation studies of CSF candidate proteins as DM biomarkers were performed by comparing the specific CSF protein levels in DM with a range of neurological conditions. For CSF proteins with perturbations associated with DM, further assessment of protein levels was conducted in plasma. The comparison of protein mRNA expression between controls and DM was performed by reverse-transcriptase PCR (RT-PCR). In addition, the protein distribution in controls and DM spinal cord tissues were assessed by immunohistochemistry (IHC). The RT-PCR and IHC analyses were performed using archival tissue material.

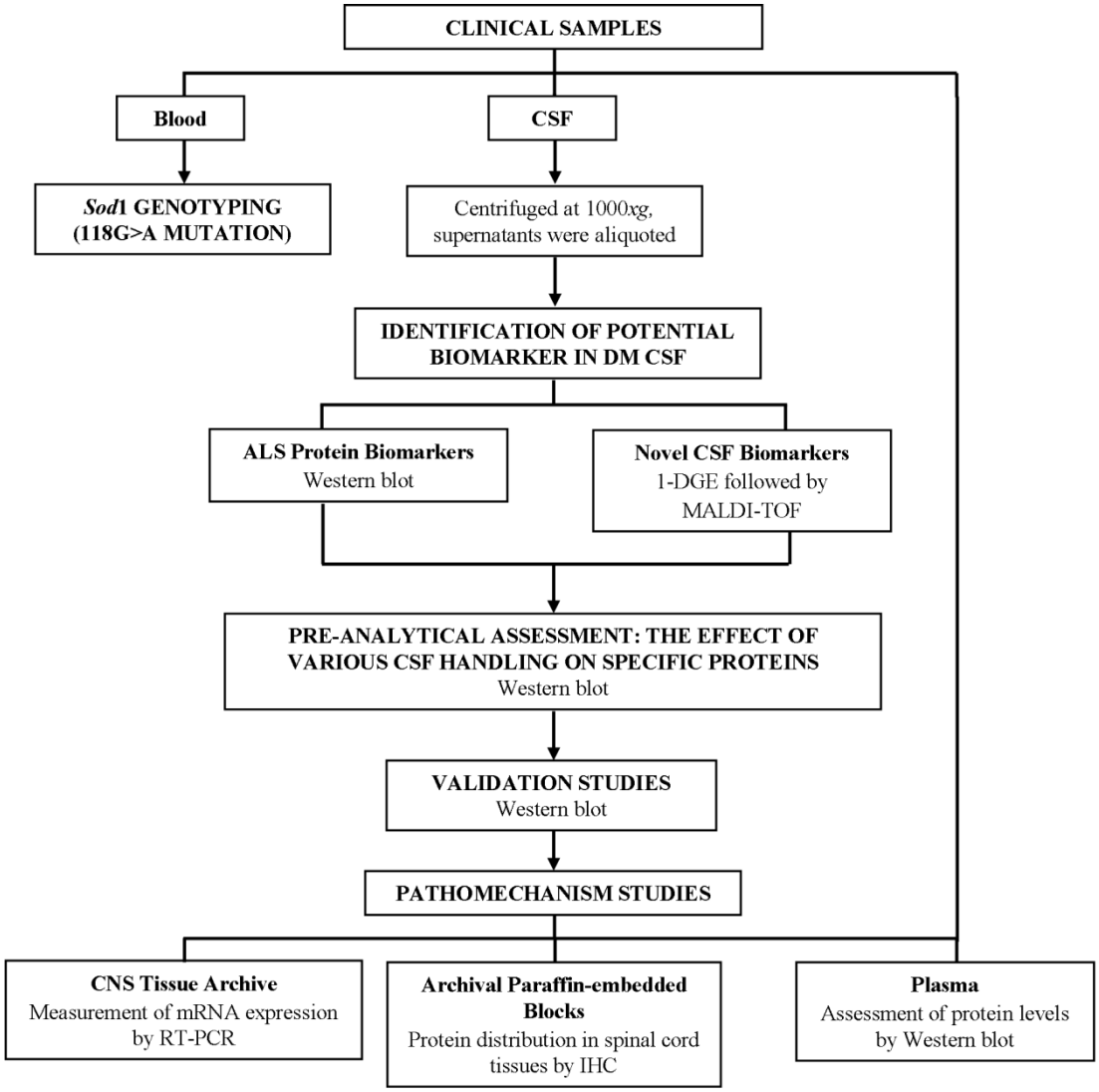

Figure 2-1: Research design and methodologies.

2.2 Case Selection

2.2.1 Clinical Material

Clinical material for this research was derived from cases undergoing routine clinical investigation at neurology service of the UGSAH. The collection of clinical material and routine clinical investigations for each case was performed by residents that were supervised by board-certified neurologists. All samples were collected with owner's permission and written informed consent (Appendix 8.5.5) following explanation on the sample deposition for diagnostic services and the use of left-over excess. A complete case history was obtained from the owner and referring veterinary surgeon and all relevant information gathered and documented by attending neurologists. A thorough physical examination was performed in all cases prior to a detailed neurological examination.

2.2.2 Clinical Diagnosis

2.2.2.1 Neurological Examination

All cases received a complete neurological examination as detailed by Lorenz and others (2004). Evaluation of mentation and changes in behaviour was based on clinician assessment at the time of anamnesis in the context of historical information. The gait was evaluated for lameness, coordination and weakness. Clinical evaluation on the cranial nerves I-XII was carried out on every patient.

Conscious proprioception and motor function were assessed by paw position, reflex stepping, hip sway test and wheel-barrowing (Griffiths and Duncan, 1975). To assess the reflex step, the paw of one limb is placed on a piece of paper, or similar material, and the paper drawn laterally, moving the paw with it. In a normal dog, this displacement of the limb results in a reflex step returning the limb to approximately the original position. Hip sway reaction is another proprioceptive test, it is carried out by holding the dog in the pelvic region and gently pushing the dog in an alternating lateral fashion. The reaction of a normal dog involves the development of ipsilateral muscle tone to maintain balance, whereas the abnormal dog may step laterally or potentially fall in the direction it is pushed. Spinal reflexes (patellar, limb withdrawal) were assessed in each limb, with the dog in lateral recumbency. Muscle tone was assessed by passive flexion and extension of the limb. Muscle bulk and symmetry were evaluated by palpation. Sensory perception as a test

of spinal cord function was assessed by application of stimuli to the pelvic limb paw. Bladder and bowel function were evaluated based on history and perineal reflex.

Based on neurological findings, the lesion(s) were localised justifying further ancillary diagnostic tests in the hospital.

2.2.2.2 Ancillary Tests

All clinical samples were submitted to Veterinary Diagnostic Services (VDS) in University of Glasgow. Blood in EDTA was used for routine haematology whereas for biochemical analysis, blood was collected in an anti-coagulant tube containing heparin. Blood smear and complete blood count (CBC) were requested for each sample. A full biochemical profile on blood included alanine transferase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), urea, creatinine, total protein, albumin, globulin, albumin-globulin ratio, bilirubin, electrolytes, calcium, phosphate, cholesterol, triglyceride, and glucose. Serum samples were also collected for potential serology tests.

CSF samples were collected routinely in conjunction with myelography or MRI procedures to assess potential involvement of the central nervous system. Assessment of the physical characteristics of the CSF was performed immediately after collection and the result was recorded. Total erythrocyte and leukocyte counts were performed using a haemocytometer slide. Microscopic examination of CSF cytospin was performed to determine the differential leukocyte count and to identify the presence of any type of pleocytosis or abnormal cells such as bacteria and neoplastic cells. Protein content of CSF was estimated by turbidimetric method using 3% sulfosalycylic acid (Cecil Instrument, UK).

Following sample collection, diagnostic imaging procedures, either radiography, myelography, CT or MRI were carried out under general anaesthesia. The precise anaesthetic regime depended upon the procedure and individual patient requirements. Electrophysiology studies such as EMG and NCV were also conducted under general anaesthesia as necessary. The tentative diagnosis was achieved based on history, clinical signs and supportive clinical findings. These details were recorded in the UGSAH electronic patient record (EPR), which was easily accessible. Cases were monitored, through re-examination, on a regular basis throughout the course of their disease.

2.2.3 Collection of Clinical Material

Whole blood (1-1.5ml) was harvested from jugular vein using 23G hypodermic needle attached to 5ml syringe. The hair over one jugular groove was clipped if necessary and the venipuncture site was aseptically prepared. The dog was restrained as appropriate with the head tipped back exposing the jugular groove. The vein was raised and the needle was inserted with the tip upwards. Blood was withdrawn by gentle suction and transferred into tubes containing EDTA.

CSF collection in dog was performed under general anesthesia. The volume of CSF taken from each subject in this study varied according to the body weight of the dog. A range from 0.5 to 1.5ml was collected into a plain tube and was sent for routine investigation as described in 2.2.2.2, page 77. The hair on the region of interest was clipped and the area aseptically prepared. For CSF collection from cistern magna, the dog was placed in lateral recumbency and the head positioned at 90° angle to the neck. The occipital protuberance and the lateral wing of atlas were identified and used as landmarks. The hypodermic needle (21G for large dog, 23G for small dog) was inserted 90° to the halfway of an imaginary midline between the occipital protuberance and the atlas (C1). Drops of CSF were allowed to flow into a plain collection tube. CSF collection from lumbar cistern was performed between the L5 and L6 or L6 and L7 vertebral interspace. The dog was positioned in lateral recumbency. The midline was located by palpating the dorsal spinous processes and the wings of ilium. A spinal needle was used for lumbar cistern collection. The stylet was removed to collect CSF. The site of CSF collection and the storage time were recorded for each sample.

All clinical samples were temporarily stored at -20°C before being transferred to laboratory storage at -80°C. The immediate storage at -80°C was not possible due to the distance of the hospital from the lab and the difficulty in predicting the exact time that cases would be examined or samples collected.

2.3 Protein Analysis

2.3.1 Sample Preparation

2.3.1.1 CSF

Following the collection of CSF into sterile tubes, samples were transferred immediately to -20 °C for short term storage (3 days). CSF samples were then transferred to the laboratory, on ice, allowed to thaw then centrifuged at 1000*xg* for 10 minutes at 4°C to remove cellular debris. CSF supernatant was aliquoted into a fresh 1.5ml eppendorf tube. The supernatant and pellet fractions were stored separately at -80°C until required. .

2.3.1.2 CNS Tissue Homogenates

A range of CNS tissue homogenates from mouse, sheep, and dog served as controls and were compared to canine CSF. Brain tissue homogenates from mouse were provided by Dr. Mark McLaughlin from Applied Neurobiology Group, School of Veterinary Medicine, University of Glasgow. Canine brain tissue was obtained during post mortem by Dr. Pamela Johnston and snap frozen in liquid nitrogen before transfer to -80°C. Prior to tissue homogenisation, the homogenisation buffer (Appendix 8.2.1) supplemented with a protease inhibitor cocktail (Sigma Aldrich Ltd, UK) was pre-chilled on ice. 0.2-0.3g of brain tissue was transferred to the pre-chilled mortar with liquid nitrogen. The tissue was briefly powdered with mortar and pestle, transferred to a 15ml tube and homogenised for 10 seconds in homogenisation buffer by 12 passes of an Ultra-Turrax T8 blender (IKA Labortechnick, USA) set at maximum speed. The homogenates were centrifuged at 1000rpm for 15 minutes at 4°C to remove debris and the supernatants aliquoted in fresh 1.5ml eppendorf tubes and stored at -80°C until required.

2.3.2 Total Protein Measurement for Biomarker Analyses

The total protein content was determined with a BCA (bicinchinonic acid) based protein assay kit (Fisher Scientific, UK) using bovine serum albumin (BSA) as protein standard. This method is an adaptation of Lowry-based method (Smith et al. 1985), which is based on the principle that the peptide bonds in aromatic amino acids reduce copper 2+ ions to cupric ions (Cu+) at an alkaline pH that then generates a purple chromogen, that is proportional to the amount of protein at a specific absorbance of 562nm. 50µl of blank (ultrapure water) and each BSA standards; 0.05, 0.1, 0.2, 0.4, 0.6, 0.8 (mg/ml) were

measured compared to 5µl from the unknown sample. Incubation of the protein mixture was conducted at 37°C for 30 minutes following addition of 1ml BCA reagents. The absorbance was read on a standard spectrophotometer (Cecil Instruments, UK). The details of BCA reagents are given in Appendix 8.2.2. The protein concentration was determined using a standard curve that was plotted based on the BSA standards (absorbance versus concentration in mg/ml) and taking the dilution factor into consideration.

2.3.3 Acetone Precipitation

Acetone precipitation was used in specific studies. CSF was desalted using acetone precipitation technique to remove albumin and to enrich any proteins of lower abundance. The volume of CSF containing a known amount of protein (typically 250µg) were mixed with 4X sample volume of 100% ice-cold acetone and incubated at -20°C overnight. The mixture was centrifuged at 13000rpm for 10 minutes. The pellet was washed twice with 80% acetone and the centrifugation process repeated. The protein pellet was allowed to dry for five minutes at room temperature. Re-suspension of protein pellet was accomplished in ultrapure water at the desired concentration.

2.3.4 Sample Denaturation and Loading

The amount of protein was calculated based on the protein concentration determined in BCA protein assay. Samples were prepared to give an equivalent amount of protein in the same volume by mixing the appropriate volume of CSF with 3X sample denaturation buffer (Appendix 8.2.3) containing sodium-dodecyl-sulphate (SDS) and made up to a final volume with ultrapure water. Samples were subsequently denatured in water bath at 90°C for four minutes. This process linearises the protein and the association of SDS induces a negative charge on the proteins that facilitates migration towards the anode on a polyacrylamide gel. The samples were loaded onto precast polyacrylamide gels with size markers (3-188kDa, SeeBlue®, Invitrogen, UK)

2.3.5 One-dimensional Gel Electrophoresis

One-DGE separates the proteins based on their molecular weight. Precast polyacrylamide gels (10 or 12 well) were employed using 1.0mm thick, 4-12% Bis-Tris mini gel (NuPAGE® Novex®, Invitrogen, UK). Electrophoresis was performed using the Invitrogen minigel rig with commercially obtained 2-(N-morpholino) ethanesulfonic acid (MES)

buffer at 1:20 dilution with ultrapure water (Invitrogen, UK). Protein separation was achieved by applying an electrical field for one hour at 140V, 250mA.

2.3.6 Gel Staining

The gel was transferred into a tray and washed three times with ultrapure water. Two staining methods were employed; Coomassie blue (SimplyBlue™ SafeStain, Invitrogen, UK) which is compatible with MS analysis and the more sensitive silver staining kit (SilverXpress®, Invitrogen, UK). The stained gels were dried for one hour using a gel dryer (BioRad Life Sciences, UK).

For the Coomassie blue stain, 20ml of solution was poured into the gel tray. The gel was agitated gently and stained for one hour. De-staining with ultrapure water was carried out three or four times, allowing 30 minutes for each wash. Visualisation of proteins by the Coomassie blue system reflects strong non-covalent binding with the proteins, which is proportional to the amount of proteins in the specific band.

Silver staining was performed using a commercially available kit as recommended by the manufacturers. Initially the gel was fixed with a solution of methanol and acetic acid in ultrapure water. Following fixation with methanol and acetic acid, the gel was incubated with sensitising agent, glutaraldehyde. Subsequently, the gel was impregnated with silver nitrate solution and developed by formaldehyde and citric acid solution. The basic mechanism involves formation of silver ions complexed with amino acid chains, which subsequently reduces into metallic silver by formaldehyde in an acidified environment, allowing the proteins to be visualised.

2.3.7 Western Blot

The Western blot technique is an antibody based technique that is widely used subsequent to 1-DGE to detect specific proteins. The proteins in the gel are transferred to nitrocellulose membrane through application of electric field for a specific period of time. The bands are visualised using a chemi-luminescence (ECL) substrate that causes oxidation of the horse radish peroxidise labelled antibody complex a process that emits light as the reaction decays to the ground state, and which is proportional to antibody binding.

Each Western blot analysis in this study was performed once, therefore the reproducibility of this technique in determining the specific protein levels were not assessed. Following electrophoresis, the gels were removed from their plastic plates, the wells were removed and the gels carefully placed onto nitrocellulose sheets that are supplied as a component of a dry blotting system from Invitrogen (iBlot®). Proteins were then transferred using this system as per manufacturer's instructions. This is a rapid procedure which is highly efficient and a reproducible transfer occurs in seven minutes. To confirm that the transfer is successful, the blot was stained with 0.1% Ponceau S in 5% acetic acid (Appendix 8.2.4), a water soluble stain which highlights the separated proteins and allows a visible inspection of loading. The nitrocellulose membrane was washed three times for 5 to 10 minutes and blocked with 5% milk powder in 1X Tris-buffered saline containing Tween 20 (1X T-TBS, Appendix 8.2.5) for one hour. The membrane was probed overnight at 4°C with primary antibody and subsequently incubated with horseradish peroxidase-linked (HRP) secondary antibody for one hour. The dilutions of primary and secondary antibodies were made in 5% milk powder in 1X T-TBS. The proteins identified in this project with their source of primary antibodies, range of dilutions and secondary antibody links are summarised in Table 2-1.

Primary antibody	Dilution	Sample	Secondary antibody	Source
Cystatin C	1:1000	CSF	HRP anti- rabbit	US Biologicals, USA
TTR	1:10,000	CSF	HRP anti-rabbit	Sigma, UK
Clusterin	1:50,000 1:100,000	CSF Plasma	HRP anti-goat	Abcam, UK
Haptoglobin	1:1500	CSF	HRP anti-goat	ReactivLab (Prof. P.D. Eckersall)
VGF	1:200	CSF	HRP anti-goat	Santa Cruz Biotechnology, UK
7B2	1:500	CSF	HRP anti-rabbit	Enzo Life Sciences, UK

Table 2-1: The list of proteins identified in this project.

Details on the source of primary antibodies, range of dilutions and secondary antibody links for Western blot were given in the table.

2.3.7.1 ECL Detection and Signal Quantification

For ECL detection, the nitrocellulose membrane was incubated with 1.5ml ECL working solution (Thermo Fisher Scientific, UK) for one minute. The membrane was removed from

ECL solution, dried with absorbent tissue to remove the excess liquid, and subsequently placed in a plastic sheet protector or clear plastic wrap. The membrane was exposed to hypersensitive film (Hyperfilm™ECL™, Amersham Biosciences, UK) at different time interval and the film was developed in an automated X-ray processor (Xograph Healthcare, UK).

The immune-complex signals visualised on the hyperfilm was scanned and digitised (Epson, UK) for Scion Image NIH software. The software generates a plot profile of the grey levels that represents the relative density over the area of the band. The area under each peak was quantified and the values generated were expressed as optical densities that were subsequently recorderd for statistical analysis.

2.3.8 In-gel Trypsin Digestion for Coomassie-stained Proteins

The gel bands of interest were identified from 1-DGE analysis and were excised for MALDI-TOF MS analysis. The excised gel bands were cut into several pieces and placed in 1.5ml eppendorf tubes. Gel pieces were washed for 45 minutes in 500µl of 100mM ammonium bicarbonate and the wash was discarded. Gel pieces were further washed in 50% acetonitrile/100mM ammonium bicarbonate for another 45 minutes. The wash was again discarded. For reduction, 150µl of ammonium bicarbonate (100mM) and 10µl of DTT (45mM) were added into the gel pieces and incubated for 20 minutes at 60°C. After cooling to room temperature, 10µl of 100mM iodoacetamide was added and the mixture was incubated in the dark room for 30 minutes. Subsequently, the gel pieces were washed in 50% acetonitrile/100mM ammonium bicarbonate for 30 minutes. After the wash was discarded, 50µl of acetonitrile was added to dehydrate the gel pieces and incubated for 10 minutes. Complete dehydration of gel pieces was completed in a vacuum centrifuge. To re-hydrate the gel, a volume of 2µg/µl sequencing grade modified porcine trypsin (Promega, UK) in 25mM ammonium bicarbonate was added. The protein solution was left to digest overnight at 37°C. Once the digest was complete, the solution was centrifuged and the supernatant was removed and stored. Elution of the peptides from the supernatant was performed at room temperature initially in 5% formic acid and subsequently in 20µl of acetonitrile both for 20 minutes. The protein extract was briefly centrifuged and supernatant transferred and completely dried in a vacuum centrifuge. The dried samples were analysed using the MALDI-TOF mass spectrometer 4700 Proteomics Analyzer (Applied Biosystems, UK). The monoisotopic peptide mass fingerprinting data generated by MS were analysed using Mascot (Matrix Science, USA), which reports all significant

hits from the SwissProt database. Statistical confidence level of 95% was applied for protein identification.

2.4 Nucleic Acid Handling

2.4.1 Extraction of Genomic DNA (gDNA)

gDNA was isolated from two different sources; blood (cases related to CSF biomarker analysis) and spleen (cases related to mRNA and immunohistochemistry). gDNA from 100µl of whole blood sample was extracted using a commercially available kit (DNeasy® Blood and Tissue, Qiagen, UK) following manufacturer's recommendations. Canine anti-coagulated blood was mixed with proteinase K (600mAU/ml/reaction) and lysis buffer containing guanidine hydrochloride to digest proteins and release nucleic acids. The mixture was incubated at 56°C for 10 minutes, followed by the addition of 100% ethanol centrifuged through a spin column, washed twice using buffers containing ethanol and guanidine hydrochloride, eluted in 200µL elution buffer (10mM Tris-hydrochloric acid (Tris-HCl), 0.5mM EDTA, pH 9.0). All buffers were supplied by the kit manufacturer.

For spleen gDNA, approximately 10mg of tissue was added to the lysis buffer and proteinase K, and incubated for two hours. Following lysis, the procedure was as for blood DNA extraction as described above.

2.4.2 Extraction of RNA

2.4.2.1 Spinal Cord Tissue Material

All spinal cord tissues were collected during post mortem conducted by Dr. Pamela Johnston between the period of 1994 and 1998. Cases were originally from referred veterinary practices across United Kingdom. Spinal cord tissues had been snap frozen and stored in liquid nitrogen until required. RNA was extracted from T12 spinal cord.

2.4.2.2 Extraction of RNA

The extraction of RNA from tissue using RNAsol Bee is a modification of a one step procedure described by (Chomczynski and Sacchi, 1987) and was conducted following manufacturer's recommendations (AMS Biotechnology, UK). RNAsol Bee contains a monophasic solution of guanidine hydrochloride and phenol chloroform. Guanidium

rapidly inactivates RNase and forms complexes with RNA and water, allowing RNA to be retained in the aqueous phase, while DNA and proteins separate in to the phenol or chloroform phase.

500µl of RNAsol Bee was pre-chilled and added to prepared tissue powdered in liquid nitrogen, using a chilled pestle and mortar. The spinal cord tissue was homogenised by passing through hypodermic needles of decreasing size (23-14G) until the RNAsol Bee was clear. The mixture was aliquoted into a fresh 1.5ml tube. Chloroform was added to each sample, which were vortexed for 10 seconds and placed on ice for five minutes. The samples were centrifuged for five minutes at 13000rpm at 4°C. The upper aqueous phase was transferred to a fresh tube. An equal volume of 100% isopropanol was added to the aqueous phase and incubated for 10 minutes. The samples were centrifuged for 15 minutes at 13000rpm at 4°C and the upper aqueous phase was transferred to a fresh tube. An equal volume of 100% isopropanol was added and samples were centrifuged for 15 minutes at 13000rpm at 4°C. The supernatant was discarded and an equal volume of 70% ethanol was added to each sample. Samples were vortexed and centrifuged for eight minutes at 13000rpm at 4°C. The supernatant was again discarded and centrifugation step was repeated for two minutes and excess alcohol removed with a pipette and micro tip. The residual pellet was left to air dry for no more than 10 minutes and re-suspended using 150µl PCR water. All extracted RNA was kept at -80℃ until further analysis.

2.4.3 Quantification of Nucleic Acids

Quantification of nucleic acids was determined by ultra violet absorbance using a GeneQuant RNA/DNA calculator (Pharmacia Biotech, UK). Nucleic acids were diluted in ultrapure water; 1:2 dilution for gDNA, 1:10 dilution for RNA. Blank ultrapure water was also prepared. Samples were read at absorption of 260/280nm on GeneQuant (Pharmacia Biotech, UK). The assessment of the ratio at 260/280nm was used as an index of the purity of the nucleic acids and a typical ratio of 1.5 to 1.6 was observed for most of the samples which is acceptable.

2.4.4 Agarose Gel Electrophoresis and Image Capture

Routine analyses of gDNA, RNA and PCR products were performed in ethidium bromide stained agarose gel at a various concentrations (0.8% to assess the integrity of gDNA or 2-2.5% to visualise RNA and small PCR products. Gels were prepared by heating a solution

of ultra pure electrophoresis grade agarose (Invitrogen, UK) in 1X Tris-acetate EDTA buffer (1X TAE, Appendix 8.3.1) in a microwave oven. The solubilised gel solution was cooled and 15µl ethidium bromide (1mg/ml) was added and the liquid gel poured into a gel rig with the appropriate size of comb. Samples were loaded with 3µl of DNA loading dye (Invitrogen, UK) and electrophoresed in 1X TAE at 70V for 30 minutes to check for nucleic acid integrity. Visualisation of DNA products was performed under ultraviolet light (GeneFlash, Syngene, UK). The gel images were photographed using UV image capture system (GeneFlash, Syngene, UK)

2.5 Tissue Morphological Analysis

All works presented in this section were carried out at the VDS, School of Veterinary Medicine with help from Mrs. Lynn Stevenson.

2.5.1 Slide Coating

Clean glass slides (Fisher Scientific, UK) were coated with mixture of 7ml of silane-based solution (Vectabond™, Vector Labs, UK) in 350ml of 100% acetone for five minutes. Elimination of excess reagent was undertaken by dipping the slides for 30 seconds in distilled water and subsequent drying overnight in an oven at 37°C.

2.5.2 CNS Material and Paraffin Processing

2.5.2.1 Fresh Spinal Cord Tissue Specimen

The spinal cord was removed by routine procedure used in the post mortem. The fresh spinal cord tissues were immersion-fixed in 4% buffered neutral formaldehyde (Appendix 8.4.1) for a minimum of 24 hours. The spinal cord was suspended and weighted to prevent curling during fixation. The spinal cord tissues were processed for paraffin wax embedding using the Tissue-Tek® VIP® (Sakura, USA) processor on a pre-set programme (Appendix 8.4.3). Paraffin-embedded tissue sections were cut at 4µm thickness with a microtome (Shandon Finesse®, Thermo Scientific, UK) and mounted onto the silane-coated slides. The slides were dried at 60ºC for an hour and were baked at 37°C overnight.

2.5.2.2 Archival Paraffin-embedded Blocks

See 6.3.3.1, page 169.

2.5.3 Haematoxylin and Eosin (H&E)

All sections were routinely stained with H&E for routine assessment of tissue morphology. Gill's haematoxylin can be oxidised to hematein that forms a complex with a mordant that selectively stains chromatin. Sections were taken "down to water" by removing the paraffin wax in histoclear followed by 70% absolute alcohol and 70% methylated spirit before rinsing in water (Appendix 8.4.2). Following hydration, sections were immersed in Gill's haematoxylin for five minutes and rinsed in water for 30 seconds. Sections were dipped for 10 seconds in 1% acid alcohol (1% HCl in methylated spirit) to eliminate excess haematoxylin and to enhance cellular differentiation. Following washing in running tap water, sections were blued in Scott's tap water substitute (Appendix 8.4.4) and subsequently dipped in eosin for five minutes. Sections were dehydrated (Appendix 8.4.2) with 70% alcohol and 70% methylated spirit, cleared in Histoclear and mounted in DPX (Cellpath, UK).

2.5.4 Immunohistochemistry

See 6.3.3, page 169.

2.5.5 Image Capture

Images of the sections were captured by using Cell^D imaging system (Olympus Soft Imaging Solutions, Germany).

2.6 Statistical Analysis

In all studies, statistical analyses and graphs were analysed by using GraphPad Prism 4.0 (GraphPad Software, USA). Statistical analysis was performed using non-parametric tests using either the Mann-Whitney *U* or Kruskal-Wallis analysis of variance (ANOVA) test with a significance level (α) set at 0.05. The choice of statistical method utilised in each study is discussed in more detail in the specific chapters.

3 The Development of a Genotyping Protocol to Identify a Point Mutation (118G>A) in the Canine *Sod*1 Gene

3.1 Background

The human superoxide dismutase 1 gene (*SOD*1) (Accession number: NG_008689.1), located on the long arm of chromosome 21q22.11, encodes the cystolic antioxidant enzyme SOD1 (Green et al. 2002). The gene consists of five exons, encoding 154 amino acids. The gene is ubiquitously expressed and the corresponding protein catalyses the dismutation of superoxide radicals to hydrogen peroxide and oxygen (Bannister et al. 1991; Stewart, 2005). Mutations in *SOD*1 gene has been classically associated with familial ALS, accounting for 20% of total familial ALS cases (see 1.3.1, page 42).

The canine *Sod*1 gene (Accession number: NC_006613.3), located at chromosome 31, shares 83% and 79% similarity at the nucleotide and amino acid respectively compared to human gene and protein (Green et al. 2002). A genetic study has established a point mutation in exon two of canine *Sod*1 gene (118G>A) in 96% of DM cases examined, resulting in glutamic acid to lysine (E40K) amino acid substitution (Awano et al. 2009), implying DM is potentially orthologous to ALS (see 1.2, page 40). In ALS, information on genetic status has enabled patients' stratification which significantly enhances diagnosis and clarifies prognosis (Andersen et al. 1996; Andersen et al. 2003). A similar situation exists in DM where the discovery of a *Sod*1 mutation has made an important contribution in the context of genetic epidemiology in DM population and at the same time raises hypotheses on potential underlying mechanisms. Although this mutation is not specific to DM, this identification has significantly improved the clinical diagnosis and has been used as a genetic marker for DM. In this chapter, we intended to use genetic information from a previous study (Awano et al. 2009; Coates and Wininger, 2010) in conjunction with clinical findings to establish a homogenous disease group with a specific genotype that would strengthen the biomarker analysis. Therefore, we developed an "in-house" protocol using a restriction fragment length polymorphism assay (RFLP) based on the abolition of an enzyme restriction site in the mutant *Sod*1 gene. However, since there is a patent covering the use of this sequence, Professor Joan R. Coates from University of Missouri was informed prior to the development of our genotyping protocol.

3.2 Aims

The aims of the work presented in this chapter were to:

1. Develop an "in-house" genotyping protocol based on the presence of the 118G>A mutation in canine *Sod*1 gene that would assist in DM case selection for biomarker analyses.

2. Provide information on genotypic distribution in the population studied.

3. Appraise the clinical information of DM-affected dogs with 118G>A *Sod*1 mutation (DM homozygote).

3.3 Materials and Methods

3.3.1 Primer Design

The canine sequence spanning the *Sod*1 mutation was interrogated for the presence or absence of specific restriction enzyme site(s) between the wild type *Sod*1 gene (WT) and mutant form of the canine *Sod*1 gene. The restriction enzyme site, *Hpy*AV was identified in wild type but absent in the mutant gene (Figure 3-1) using NEBcutter V2.0 (Vincze et al. 2003). The forward (5-/ GCC TGT TGT GGT ATC AGG AAC CA-3/) and reverse (5/-AGA GTC AAA AAC CGG C TT TGT GGA-3/) *Sod*1 primers (Eurofins, Germany) were designed to amplify this region, using an interactive web-based primer program algorithm, GeneFisher software version 1.2.2 (BiBiServe, Germany). These primers generate a 236 base pair (bp) DNA fragment encompassing the point mutation and the diagnostic *HpyAV* restriction site. A *Sod*1 genotyping protocol to differentiate wild type (G/G), heterozygous (A/G) and homozygous (A/A) individuals using RFLP technique is summarised in Figure 3-2.

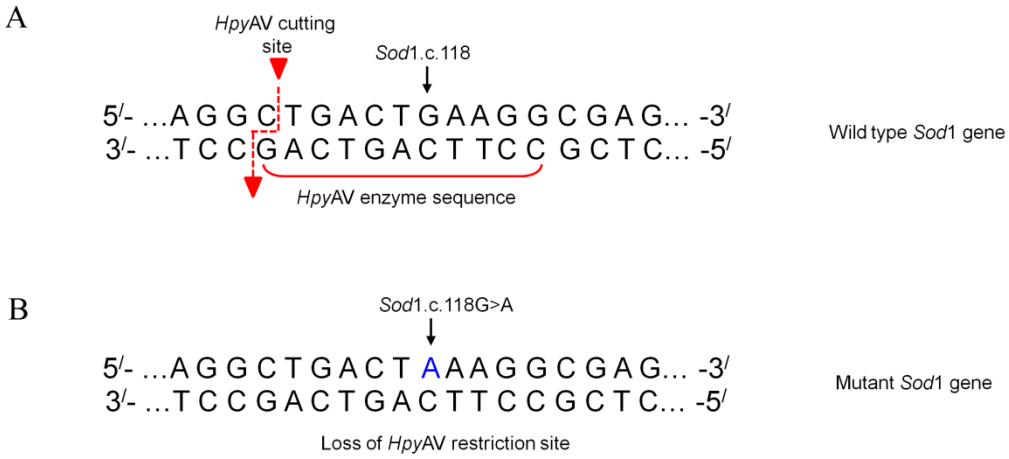

Figure 3-1: Nucleotide sequences of A) wild type *Sod*1 gene containing the *Hpy*AV restriction site and B) mutant *Sod*1 gene showing loss of the *Hpy*AV site.

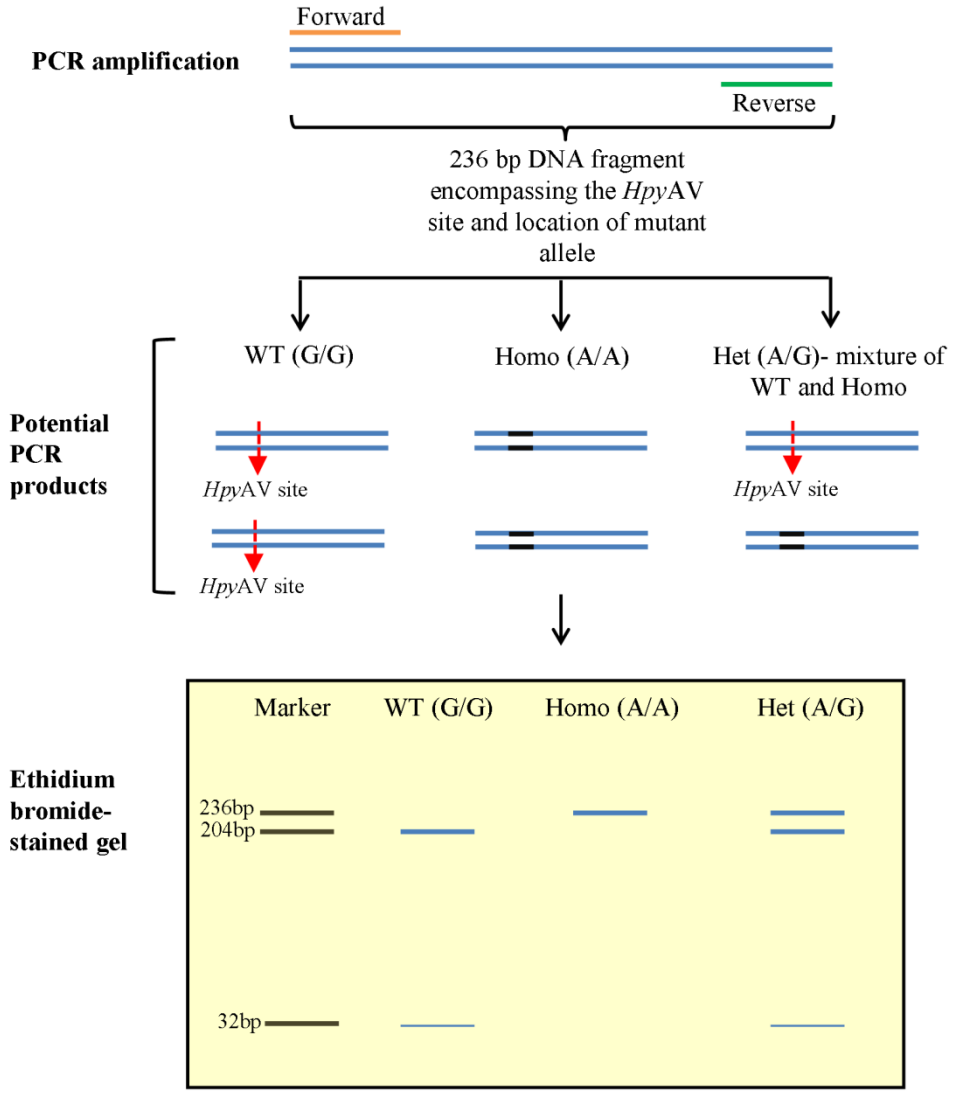

Figure 3-2: The schematic diagram of *Sod*1 genotyping using RFLP technique.

Interrogation of canine *Sod*1 gene sequence in exon two revealed a *Hpy*AV restriction enzyme site which is absent in the mutant form. Forward and reverse primers were designed to generate a 236bp DNA fragment harbouring the G>A mutation also containing *Hpy*AV site that is present in the wild type (WT) gene but absent in the mutant gene (Homo). The digestion products can be visualised in ethidium bromide-stained agarose gel. *Hpy*AV digestion cuts the wild type gene into two DNA fragments; 236bp and 32bp. As the restriction site is absent in the mutant *Sod*1 gene, there is no digestion and a single band can be visualised at 236bp. Heterozygous (Het) individuals express both wild type and mutant *Sod*1 genes revealing three bands at 236bp, 204bp and 32bp.

3.3.2 gDNA Preparation

Two different sources of gDNA were used; blood and spleen (see 2.4.1, page 84). All gDNA were quantified as described in 2.4.3 (page 85) and qualitatively assessed for degradation by electrophoresis using low percentage (0.8%) agarose gel (see 2.4.4, page 85).

3.3.3 Polymerase Chain Reaction (PCR)

A total of volume of 25µl PCR reaction was prepared, comprising 12.5µl RedTaq® DNA polymerase buffer (Sigma-Aldrich Co, UK), 0.5µl of each primer (5pmol per reaction tube), and 200ng gDNA. Amplification conditions were 32 cycles (94°C for five minutes, 94°C for one minute, 58°C for one minute, 72°C for one minute, 72°C for 10 minutes). The PCR products (2µl) were visualised using ethidium bromide (1mg/ml) stained agarose gel and quantified against the 100bp mass ladder for DNA (Quick-Load, New England Biolabs, UK).

3.3.4 PCR Product Purification and Quantification

Purification of PCR products (pooled triplicates) was according to manufacturer's recommendations using the QIAquick® PCR purification kit (Qiagen, UK). In brief, after adding the DNA-binding buffer (guanidine hydrochloride and isopropanol) to the PCR sample, the mixture was transferred into a spin column and centrifuged at 13000rpm for 60 seconds. The PCR product were washed twice and eluted in 30µl of elution buffer. The column was left standing for one minute at room temperature and centrifuged as described previously. The purified PCR products were electrophoresed using 2.5% agarose gel with inclusion of 100bp DNA marker. Quantification of purified PCR products for *Hpy*AV digestion was calculated using ScionImage NIH in respect to a band in 100bp mass ladder.

3.3.5 *Hpy*AV Digestion

Digestion of purified PCR products was performed with *Hpy*AV (2U/µl) in a 25µl reaction mix comprising 100ng of PCR amplicon, 2.5µl of 10X BSA buffer, 2.5µl of 10X buffer 4 (20mM Tris-acetate, 50mM potassium acetate, 10mM magnesium acetate, 1mM DTT at pH7.9), and incubated at 37°C for 30 minutes. In addition, an undigested control was included. The reaction was heat inactivated at 65°C for 15 minutes. The digestion products were analysed by 2.5% agarose gel electrophoresis (see 2.4.4, page 85) in 1XTris-borate

EDTA (1X TBE, Appendix 8.3.2). Gel images were captured as described in (see 2.4.4, page 85).

3.3.6 Selection of DM Cases based on Clinical Findings and Genetic Status

The case materials for CSF biomarker study were sourced from neurology service, UGSAH. A complete physical and neurological examination was performed as detailed in 2.2.2, page 76. The tentative diagnoses of cases for biomarker analysis were achieved by the presence of typical clinical signs *i.e* pelvic limb ataxia, and paresis with a loss of proprioception and the exclusion of other causes through further clinical investigations. At this stage, cases for inclusion in this study were selected on the basis of historical information and clinical examination which suggested a chronic neurodegenerative disease that affect the thoracolumbar spinal cord.

All putative DM cases were subsequently genotyped with RFLP assay based on the abolition of *Hpy*AV site in the mutant *Sod*1 gene. To eliminate variation in the group analysed, only cases that had been confirmed as homozygote were included in biomarker studies. Once the DM group was established, we also evaluated the clinical information including signalments, clinical signs and neurological findings. The results were tabulated and compared with previous studies.

3.4 Results

3.4.1 Genotyping Analysis based on 118G>A *Sod*1 Mutation in Cases Studied

*3.4.1.1 Hpy*AV *Digestion of Spleen-derived gDNA*

Five cases from each non-neurological (*N*=5) and DM-affected (*N*=5) groups from our archive material were utilised to determine if the RFLP technique outlined in Figure 3-2 could differentiate between the three potential *Sod*1 genotypes; wild type, heterozygous, and homozygous. Purified PCR products generated from spleen gDNA were analysed at a digestion time of 30 minutes (Figure 3-3A). The digestion profiles are presented in Figure 3-3B. In putative DM cases (A1-A5), a single band (236bp) of equal size to the undigested sample (UD) was detected, which were consistent with homozygosity for the mutant allele.

Four control cases (C1-C4) demonstrated a profile of a doublet composed of a weak band at 236bp and a strong band at 204bp, which were consistent with the lack of mutation, albeit with evidence of partial digestion. One control case (C5) was consistent with the heterozygous profile, demonstrated by a doublet bands corresponding to 236bp and 204bp at equal intensities. It should be noted that the size migration of these bands compared to the 50bp marker was slightly higher than their predicted size and could be attributable to the different gel buffer used for the commercial marker compared to that used for the samples.

As identifying a non-mutated allele was paramount to genotyping and with evidence of partial digestion, a titration experiment of the conditions was undertaken (Figure 3-5).

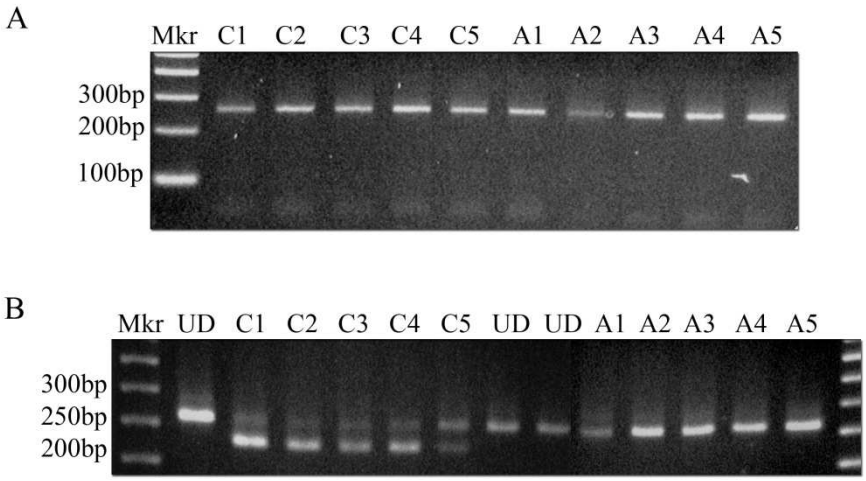

Figure 3-3: Digestion profiles of *HpyAV* on splenic DNA fragments at 30 minutes incubation

A) Purified PCR products can be visualised at 236bp and display good integrity despite the weak signal observed in a DM sample (A2) B) Following 30 minutes digestion with *Hpy*AV, all putative DM cases (A1-A5) exhibit a single band at 236bp, co-migrating with undigested control (UD). This profile is consistent with homozygosity for the mutant allele. Double bands compose of a weak band at 236bp and a strong band at 204bp is observed in four control cases (C1-C4). The weak band in these control cases suggests partial digestion. A control sample (C5) exhibits two bands at almost equal intensities at 236bp and 204bp, which is consistent with heterozygosity. Note that the migration of size of these bands compared to the 50bp marker is slightly higher than their predicted size.

3.4.1.2 HpyAV Digestion of Blood-derived gDNA

The genotyping analysis was performed in canine blood-derived DNA using the same protocol (Figure 3-4). Splenic samples representing the three different genotypes were used as reference control; C1 and C2 (wild type), A1 (homozygous) and C5 (heterozygous) (Figure 3-3). All purified PCR products derived from blood demonstrated a robust signal with good integrity through agarose gel analysis (Figure 3-4A). *Hpy*AV digestion for 30 minutes as for spleen PCR products gave similar profiles as demonstrated in Figure 3-4B. Unlike the partial digestion observed in control sample from spleen (C1 and C2), a single band (204bp) consistent with wild type profile was detected in B1 blood sample, indicating a complete digestion had occurred with 100ng of PCR product. This suggested that 30 minutes incubation is optimum for blood-derived DNA. The heterozygous profile was detected in B3 blood sample, demonstrating two bands at almost equal intensities (236bp and 204bp) and therefore showing a clear-cut differentiation between partially digested DNA products as observed in spleen-derived DNA. Two blood samples (B2 and B4) demonstrated homozygous profile, which represented a single band with size corresponding to undigested sample (236bp). Sample B1, B2 and B3 were used as reference controls throughout the genotyping analyses.

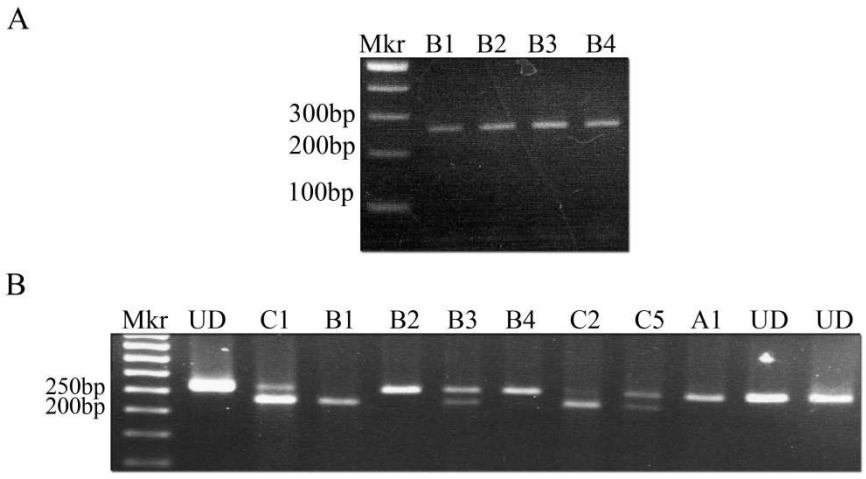

Figure 3-4: Digestion conditions of *HpyAV* on gDNA fragments from blood at 30 minutes.

A) The purified PCR products from four blood samples (B1-B4) were analysed on an agarose gel along with a 100bp DNA mass ladder., showing good integrity of PCR amplicons B) *Hpy*AV enzyme digestion of PCR products from blood and spleen at 30 minutes incubation. Spleen PCR products, C1,C2 and C5 demonstrate a consistent genotype profiles with the previous experiment (Figure 3-3B). Complete digestion is observed in B1 blood sample which is consistent with wild type profile. Heterozygous profile; two bands with equal intensities around 236bp and 204bp) is detected in B3 blood sample which can be differentiated from partially digested profile from C1 sample. B2 and B4 blood samples are consistent with homozygous profile, indicated by a single band at 236bp, which corresponds to the size of undigested control (UD).

3.4.1.3 HpyAV Titration Study

The occurrence of partial digestion could complicate the differentiation between a genuine heterozygous case (50% wild type and 50% homozygous for mutant allele) and a partially digested sample from a wild type case. Mixtures of wild type and homozygous PCR products were generated to represent each of the genotypes: wild type (100% WT: 0% Homo), heterozygous (50% WT: 50% Homo), and homozygous (0% WT: 100% Homo). Intermediate ratios were also included; 75% WT: 25% Homo and 25% WT: 75% Homo.

The result of *Hpy*AV titration experiment is shown in Figure 3-5. The heterozygous genotype displayed two bands at equal intensities (236bp and 204bp) in a mixture containing 50% wild type and 50% homozygous. In 100% wild type mixture, a prominent band was observed at 204bp suggesting DNA fragments are completely digested whereas in 100% homozygous sample, a single band was observed at 236bp, which is comparable with undigested PCR product. This confirmed the hypothesis outlined in Figure 3-2.

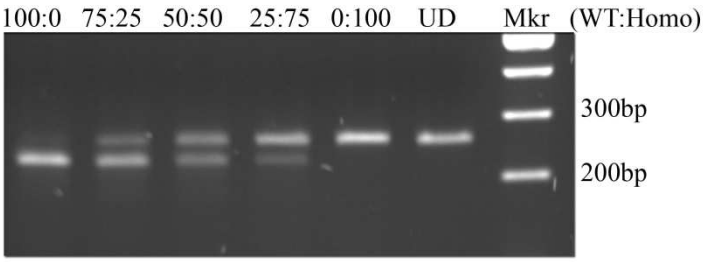

Figure 3-5: *Hpy*AV digestion of blood PCR products with different WT:Homo ratios.

The *Hpy*AV titration experiment was undertaken to differentiate a genuine heterozygous with partially digested DNA fragments in wild type profile. The mixture of wild type and homozygous PCR products at 50% WT: 50% Homo demonstrates that the expression of heterozygous can be confirmed by the presence of two bands of equal intensities.

3.4.1.4 Prevalence of 118G>A Sod1 Mutation in Clinical Cases Examined

In total, 53 cases were genotyped using spleen and blood-derived DNA (Table 3-1), representing a range of clinical diagnoses, including DM. The distribution of three *Sod*1 genotypes; wild type, heterozygous and homozygous is shown in Figure 3-6A. The percentage of wild type cases in the population analysed is 34.6% (*N*=18), whereas heterozygous cases displayed slightly higher percentage than wild type population which was 38.5% (*N*=21). Cases with homozygous profile accounts for 26.9% (*N*=14). The complete lists of dogs that were genotyped in this project are given in Appendix 8.1.1 and 8.1.2.

The distribution of three *Sod*1 genotypes in putative DM and control cases is summarised in Figure 3-6B and C. In DM cases, the homozygous population is 77.8% (N=14) which was greater than heterozygous, 22.2% (*N*=5). Analysis of the control group revealed that 52.9% (*N*=18) of cases were wild type and 47.1% (*N*=16) were heterozygous. There were no homozygous cases in the control group. The ratios are as follows; putative DM group – 0WT:4Het:14Homo, control group – 18WT:17Het:0Homo.

Table 3-2 summarises the distribution of wild type, homozygous and heterozygous genotypes in different breeds. High numbers of homozygous cases were present in the GSD breed group (*N*=13), although this may be contributed by the large number of GSDs examined in this study. One Collie dog was also confirmed as a homozygote. The ratio between three genotypes in GSD is 8WT:10Het:13Homo. A high frequency of heterozygotes were observed in Boxer dogs, where the ratio of heterozygote to wild type was 3:1 (Het:WT). In addition, a high number of heterozygotes were detected (*N*=5) in the other breed (small breed and cross breed) category, however, no homozygous cases were identified in this group.

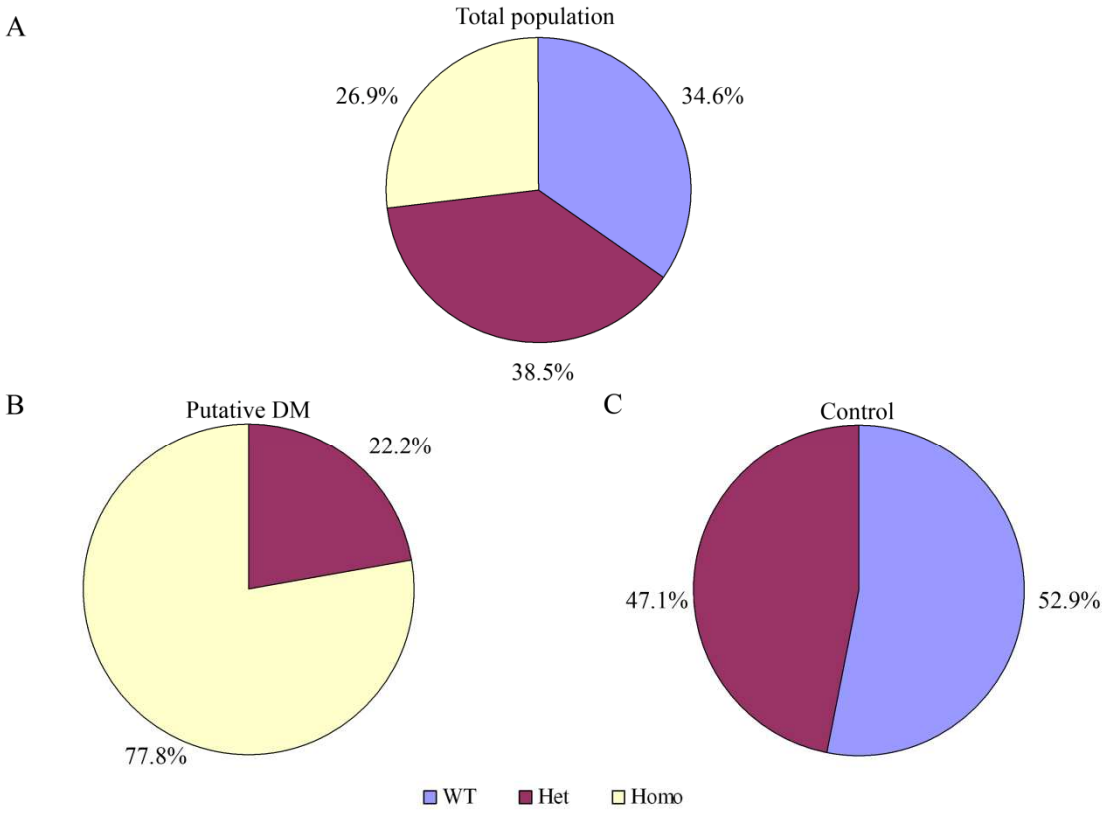

Figure 3-6: The distribution of wild type, heterozygous and homozygous in dog population studied.
A) The distribution of three genotypes in cases examined revealed 34.6% wild type, 38.5% heterozygous and 26.9% homozygous (18WT:21Het:14Homo). Heterozygote cases account for the highest percentage in this population. B) The distribution of three genotypes in putative DM group demonstrates high percentage of homozygous (77.8%) compared to heterozygous (22.2%) with a ratio of 1 Het:3.5 Homo. There are no wild type cases identified in this group suggesting that homozygous genotype is highly associated with DM. C) In control group, wild type represents 52.9% of total cases whereas 47.1% of the cases are confirmed as heterozygotes. No homozygous genotype identified in control group.

Genotype	Blood	Spleen	Total
WT	14	4	**18**
Het	20	1	**21**
Homo	9	5	**14**
	43	**10**	**53**

Table 3-1: The distribution of wild type, heterozygous and homozygous from two DNA sources; blood and spleen.

	BMD	Collie	Boxer	GSD	Doberman	Retrievers	Husky	Other breed
WT		1	1	8		3	1	4
Het	1		3	10	1	1		5
Homo		1		13				
Total	**1**	**2**	**4**	**31**	**1**	**4**	**1**	**9**

Table 3-2: The distribution of wild type, heterozygous and homozygous in DNA in a range of canine breeds.

The ratio of wild type, heterozygous and homozygous genotype in GSD examined is 8WT:10Het:13Homo. One Collie dog was also confirmed as harbouring the homozygous genotype. High number of heterozygotes are observed in Boxers, where the ratio is 1WT:3Het. High number of heterozygotes (*N*=5) with no homozygotes are also identified in other breed group, which is comprised of small breed and cross breed dogs.

3.4.2 Refinement of DM Classification based on *Sod*1 Genotyping

Prior to *Sod*1 genotyping, 18 putative DM cases were identified for this project, comprised of 17 GSDs and 1 Border Collie. Genotyping against 118G>A on these cases revealed 14 homozygotes and 4 heterozygotes. All four heterozygous cases had a presumptive diagnosis of DM based on the clinical investigations, however these dogs were excluded from further study to minimise variation in DM group.

Since the CSF and spinal cord tissue material were obtained from different sample archives and were collected at a different time, the clinical and neurologic findings are discussed and tabulated separately. The common presenting signs, the age of onset and whether there was any sex predisposition are of particular interest. The overall mean age of onset was 8.5±2.1 years with no sex predisposition (M:F=1.1.3).

3.4.2.1 CSF Biomarker Investigations

At the beginning of the research, the total number of cases identified was 13 dogs, comprised of 12 GSDs and 1 Border Collie. Following *Sod*1 genotyping, nine dogs were confirmed to be homozygous for the mutant allele. Material derived from these cases were utilised in the biomarker analyses. Four of the dogs are confirmed as heterozygotes, therefore were excluded from further studies.

Among these nine homozygote dogs, eight were GSD and one case was a Border Collie. None of these dogs were littermates. The ratio of affected males in this group was 2:1 to females. There was no record of an exact onset of clinical signs, however most owners were able to describe the duration of disease progression in general terms before first presentation at UGSAH (M±SD=18±9weeks). The mean age when first presented was eight years old (M±SD=8.5±1.9). Cases were most commonly presented to UGSAH when the ataxia and paraparesis had deteriorated to the point where they had precipitated significant owner concern leading to referral. The range in the development of clinical signs before first presentation was one to seven months. Only three dogs exhibit wearing and bleeding nails. All dogs displayed signs consistent with a non-painful pelvic limb ataxia, with weakness and various degree of paraparesis at the time of first presentation. Other problems reported by owners were difficulty ascending stairs, occasional collapse on the predominately affected side and bunny hopping. At the early stage of disease, eight cases out of nine exhibited asymmetric pelvic limb signs, 56% of dogs had right side more

prominently affected. Neither of the dogs demonstrated thoracic limb involvement or urinary incontinence. Case signalment is summarised in Table 3-3.

Signalment	**Value**
Total number of dogs	9
GSD	8
Non-GSD	1
Mean age when first presented	8.5±1.9 yo
Sex	
Male (intact)	1
Male (neutered)	5
Female (intact)	0
Female (neutered)	3
Duration of progression before first presented	
Mean	18±9 weeks
CSF protein concentration	40.2±14mg/dl

Table 3-3: The signalment findings in DM dogs examined for CSF biomarker study.

The neurological examination findings at first presentation is summarised in Table 3-4. Normal mental status and cranial nerves were reported in all dogs. Slow or absent postural reactions were observed in all dogs, which were lateralised in the majority of cases. Spinal reflexes were normal, although reduced patella and withdrawal reflexes were seen in few cases. There were no remarkable abnormalities observed on routine haematology and biochemistry. The mean of CSF total protein in all dogs was 40.2mg/dl with SD of 14mg/dl (reference range 10-40mg/dl). However CSF samples from two dogs were taken from lumbar cistern and both exhibited high CSF total protein, 60mg/dl and 58mg/dl (reference range for lumbar CSF <45mg/dl)(Di Terlizzi and Platt, 2009). Three of the dogs were found to have mild disc degeneration on MRI, however no significant spinal cord compression was detected. Two dogs also confirmed to have spondylosis at T11 to T12 vertebral segment. None of dogs in this group was euthanised or died prior to the last follow-up.

Only one dog received steroid therapy at the time sample was collected. Intensive physiotherapy and hydrotherapy were recommended to all owners. However, follow-up

was inconsistent and therefore accurate information regarding any further rate of deterioration was not obtained.

Clinical Signs	**Number of Dogs**
Normal mental status and cranial nerves	9 (100%)
Symmetry of clinical signs	
Right side more affected	5 (56%)
Left side more affected	3 (33%)
Right and left side equally affected	1 (11%)
Scuffed paws and worn nails	3 (33%)
Decreased muscle tone and muscle mass	1 (11%)
Ataxia	9 (100%)
Paraparesis	9 (100%)
Postural reactions	
Slow	3 (33%)
Absent	6 (67%)
Spinal reflexes	
Normal reflexes	5 (56%)
Reduced patella	1 (11%)
Reduced withdrawal	3 (33%)
Concurrent conditions	
Spondylosis	2 (22%)
Mild disc degeneration	3 (33%)
Therapy	
Steroid	1 (11%)

Table 3-4: The clinical signs and neurologic findings in dogs examined.

3.4.2.2 mRNA and IHC Analyses

Five DM cases (three GSDs and two GSD crosses) were selected on the basis of histopathological diagnoses. All five cases were genotyped and confirmed as homozygotes (3.4.1.1, page 94). Since these materials were obtained a decade ago, the clinical information on these dogs was very limited except for the signalments. Therefore, information on neurologic findings was not obtained, though as these dogs had met the criteria of the previous study it can be assumed a clinical diagnosis of DM was appropriate. There is no sex predilection observed (ratio M:F=1:1.5). The mean age at first presentation is 8.3±2.5 (M±SD) years old, which consistent with dogs in CSF study (8.3±2.4).

The summary of signalment findings were given in Table 3-5.

Signalment	**Value**
Total number of dogs	5
GSD	3
GSD crosses	2
Mean age when first presented	8.3±2.4 yo
Sex	
Male (intact)	2
Male (neutered)	0
Female (intact)	0
Female (neutered)	3

Table 3-5: The signalment findings in dogs examined for mRNA and IHC studies.

3.5 Discussion

Blood samples have been consistently proven to be a robust source of genetic material as it contains substantial quantities of high quality gDNA for diagnostic and research purposes (Hansen et al. 2007). For this study, we selected blood as the primary source of genetic material as it is frequently harvested in sufficient volume, however it may not be available for some cases. The collection of blood samples from client owned dogs is not permitted unless medically advised and can only be archived with the owner's consent, therefore restricting the collection of blood from control or healthy subjects (Rincon et al. 2011). Tissue material for gDNA is also robust however more difficult to obtain. Recently, the use of alternative sources of gDNA from saliva (Mitsouras and Faulhaber, 2009; Yokoyama et al. 2010) and buccal swab (Oberbauer et al. 2003; Chang et al. 2007; Rincon et al. 2011) for research and diagnostic purposes has been described and may become a preferable choice for the owner. Therefore, in future, utilisation of these alternative sources for *Sod*1 genotyping should be explored especially in large-scale studies involving DM.

We have shown that RFLP assay using *Hpy*AV enzyme digestion is robust and reliable in differentiating three distinct genotypes; wild type, homozygous and heterozygous. In total, 53 cases from various breeds were genotyped in this study as summarised in Figure 3-6. The frequency of wild type and homozygous in control group is not consistent with the previous studies where both genotypes have been reported in both control and DM group (Awano et al. 2009; Adams et al. 2010). The distribution of homozygous in control group in the previous studies were 34% (Awano et al. 2009) and 24% (Adams et al. 2010), whereas wild type cases represented as 2% (Awano et al. 2009) and 14% (Adams et al. 2010). Interestingly, the homozygous genotypes were only detected in putative DM cases (14/18 cases - 77.8%), with a high number of homozygous in affected GSD (*N*=13). Awano and colleagues (2009) have reported that 96% of DM-affected dogs (from five breeds) were homozygous for the mutant allele. Four out of five GSD in the same study were also confirmed as homozygotes (Awano et al. 2009). In addition, a *Sod*1 genotyping study on GSD population revealed that 76% of the DM-affected GSD were homozygotes (Adams et al. 2010). Based on these observations, the high frequency of mutant allele homozygosity in GSD may indicate the potential for high penetrance in this breed. There are no wild type dogs identified in the DM group and although heterozygous cases are observed, this is at a lower frequency (almost four times compared to homozygous genotype; 22.2%). The heterozygous profile in DM dogs was also observed in previous

studies, although lower frequencies were reported; 9% (GSD only) (Adams et al. 2010) and 2% (Awano et al. 2009). A high frequency of the heterozygous genotype is also detected in control group, which accounts for 47.1% compared to 32% (Awano et al. 2009) and 33% (Adams et al. 2010) in previous studies. Heterozygotes have been described as the carriers of the mutant allele and can pass the mutant *Sod*1 gene to their offspring, however the actual genotype-phenotype correlation in heterozygous cases is unknown, although recent evidence has suggested that heterozygosity may reflect the subclinical condition of DM (Coates and Wininger, 2010).

The clinical signs of DM have been recognised for many years and described by several authors (Averill, 1973; Griffiths and Duncan, 1975; Johnston et al. 2001; Coates and Wininger, 2010). The clinical characteristics of homozygous dogs affected with DM for both biomarker and tissue studies are documented in this chapter (see 3.4.2, page 104). Previous studies have reported a wide range of frequencies involving DM-affected GSD and its crosses, from 6.5% to 95.5% (Averill, 1973; Griffiths and Duncan, 1975; Kathmann et al. 2006; Awano et al. 2009). In this study, the occurrence of DM in GSD and its crosses was described at high frequency, which accounts for 83%. The occurrence of DM in the Collie breed and its crosses were reported in two studies; 13.6% (Averill, 1973) and 6% (Kathmann et al. 2006). The mean age of onset in this study was reported as 8.5±2.1 years which is consistent with previous studies that range from 8.2 to 9.3 years (Averill, 1973; Griffiths and Duncan, 1975; Johnston, 1998; Kathmann et al. 2006). There was no significant sex predilection observed in affected GSDs, similar to those reported in previous studies (Averill, 1973; Johnston, 1998; Kathmann et al. 2006). The signalment and clinical findings of DM in this study correlates with the findings reported from earlier studies (Averill, 1973; Griffiths and Duncan, 1975; Johnston et al. 2000). The most common neurologic signs noticed at first presentation are ataxia and paraparesis.

The mode of inheritance of *Sod*1 mutation in DM has not been fully elucidated, although evidence is indicative of a recessive trait, which requires both copies of the abnormal A allele to develop DM. The distribution of the three genotypes in control and putative DM cases also fits the characteristic of incomplete penetrance (Awano et al. 2009; Adams et al. 2010). In ALS, familial cases associated with *SOD*1 mutations are most commonly inherited through autosomal dominant mode with high penetrance (Cudkowicz et al. 1997; Andersen et al. 2003; Battistini et al. 2010). However, recessive traits with incomplete penetrance, D90A (see 1.3.3, page 46) have been reported in familial ALS (Andersen et al.

1996; Hand et al. 2001). The recessive trait of D90A is thought to be analogous to DM, which typically starts in the lower limbs and has a slower progression compared to other *SOD*1 mutant forms of ALS. Awano and others (2009) also speculated that homozygous dogs that are free from symptoms may develop DM when they are older, suggesting the age-related incomplete penetrance. Other possibilities that have been described include the involvement of modifier loci or non-genetic factors such as environmental effects that may influence the development of DM (Awano et al. 2009). Such hypotheses merit further investigation. Discovering other causative aetiologies in DM may provide further understanding of the disease pathophysiology and facilitate effective treatments and could potentially be implicated in the causation of ALS.

Initially, all dogs which had clinical signs of DM were considered as potential candidates for inclusion in this study, however identification of their genetic status has led to further refinement of DM classification. The use of genotyping to classify DM cases has not resulted in a group that is apparently different to the accepted clinical picture. However, by using genotyping, the heterogeneity of the cases used for further studies has been reduced and a focus is given to a homogenous group with a potentially common aetiology.

4 The Characterisation of Putative ALS Biomarkers in DM CSF

4.1 Background

DM has been recognised as a spontaneously occurring animal model of ALS based on the identification of *Sod*1 mutations in DM-affected dogs (Awano et al. 2009). In ALS, *SOD*1 mutations are attributed to 5-10% of ALS cases with high variations in disease penetrance (Battistini et al. 2010), whilst the aetiology of the majority of ALS cases remains elusive (Wijesekera and Leigh, 2009). Such heterogeneity in the disease susceptibility and pathogenic mechanisms complicates the clinical diagnosis of ALS (Beghi et al. 2011). Therefore, there has been intense research efforts in the development of reliable biomarkers for ALS that are independent of genetics, aiming to improve the diagnosis of ALS (Ryberg and Bowser, 2008). To date, more than 40 CSF proteins have been evaluated for ALS biomarkers, including cystatin C, TTR, 7B2 and VGF (see 1.7, page 68).

The most promising candidates described in the ALS literatures are cystatin C and TTR proteins (Ranganathan et al. 2005; Ryberg et al. 2010; Wilson et al. 2010). Cystatin C is a non-glycosylated protein that belongs to a protease inhibitor group, which is ubiquitously expressed in tissues and body fluids including CSF (Abrahamson et al. 1986; Abrahamson et al. 1990). This protein has been suggested to modulate the extracellular proteolysis activity through inhibition of cysteine peptidases such as cathepsin molecules, B, H, L and S (Abrahamson et al. 1990). Pertubations of TTR have also been described in ALS (Ranganathan et al. 2005). TTR (formerly known as pre-albumin protein), is a homotetrameric protein complex (molecular mass of 55kDa) linked by disulphide bridge formation (Foss et al. 2005). It is a non-glycosylated protein that is present in plasma and CSF, serves as a carrier of both thyroid hormones (T_3 and T_4) as well as vitamin A in complex with retinol-binding protein (Ingenbleek and Young, 1994; Schreiber and Richardson, 1997). The structure of TTR is complex and the expression of this protein may differ between species, it can also be influenced by *in vitro* conditions such as reducing agents (Foss et al. 2005). Recent studies have also reported alterations of 7B2 and VGF proteins in ALS CSF (Ranganathan et al. 2005; Pasinetti et al. 2006). These proteins are both localised in neuroendocrine tissue (Hahm et al. 1999; Westphal et al. 1999). 7B2 is a highly conserved protein, which is involved in the activation of the prohormone convertase 2 (PC2) that drives the maturation of many polypeptide hormones and neuropeptides (Iguchi et al. 1984; Lee et al. 2006). VGF is a neuropeptide and a member of the secretogranin family of proteins, which is stored in the large dense core vesicles in neuroendocrine tissues and neuronal cells (Levi et al. 2004). The role of VGF is poorly

understood although it has been associated with the regulation of energy homeostasis, metabolism (Hahm et al. 1999; Salton et al. 2000) and synaptic plasticity (Alder et al. 2003).

Since biochemical changes in the CNS system are often reflected in the CSF, many studies have utilised CSF as a source of biomarker discovery for neurological diseases (Ranganathan et al. 2005; Tumani et al. 2009; Blennow et al. 2010). However, biomarker investigations can be compromised by various pre-analytical factors (eg., sample processing or storage) that may arise from clinical and laboratory environments (Ferguson et al. 2007). Within our institute, there is a significant geographical distance between the small animal hospital and the research facilities, therefore collection and handling of samples until storage is the responsibility of clinical and support staff. The development of a reliable protocol for this early stage of sample collection is important in the integrity of samples used for biomarker studies.

4.2 Aims

In this chapter, we present evaluation of the selected ALS putative biomarkers (TTR, cystatin C, 7B2 and VGF) in DM CSF. These candidate proteins were selected based on the recent publications on ALS biomarkers in CSF. The specific aims of the work presented in this chapter were to:

1. Validate the cross-reactivity of commercial antibodies against putative ALS biomarkers; cystatin C, TTR, 7B2, and VGF in canine CSF.

2. Assess the influence of potential sample handling practices on the candidate protein levels in canine CSF.

3. Comparative evaluations of the candidate protein levels in DM and other neurological disorders CSF.

4.3 Materials and Methods

4.3.1 Validation of Commercial Antibodies in Canine CSF

4.3.1.1 Sample Preparation

CSF material for this experiment was taken from idiopathic epilepsy (IE) cases and compared with a range of tissue homogenates from dog and mouse. CSF supernatants (see 2.3.1.1, page 79) were utilised for the experiment and the total protein concentration was determined for each CSF supernatant by BCA protein assay (see 2.3.2, page 79).

4.3.1.2 One-DGE and Western Blot

Twenty-five µg of protein was taken from CSF and brain tissue homogenates (mouse and dog), mixed with SDS buffer and denatured as detailed in 2.3.4, page 80. Protein samples were analysed by 1-DGE (see 2.3.5, page 80) and subsequently subjected to Western blot analysis (see 2.3.7, page 81). The details of commercial antibody for cystatin C, TTR, VGF and 7B2 are summarised in Table 2-1.

4.3.1.3 Optimisation of Western Blot Analysis for TTR

Initial Western blot analysis demonstrated that two TTR subunits are identified in canine CSF (Figure 4-1B). To identify the optimum protein amount and confirm the linear relationship between the protein content and signal detected by ECL a gradient of protein amounts (2.5µg, 5µg, 10µg, 20µg) of IE CSF were compared by Western blot for TTR. The signals generated from the ECL reaction were quantified for each subunit and arbitrary values were plotted in XY scatter graph. As both subunit bands have noticeably different intensities on Western blot, the image capture parameter (exposure time of blot to hypersensitive film) was also optimised for each band.

For the other antibodies a standard 5µg protein amount was utilised for each Western blot.

4.3.2 Pre-Analytical Assessment: Influence of Sample Handling Regimes on the Candidate Protein Levels in Canine CSF

4.3.2.1 Source of Material and Simulated Clinical Conditions

Ten CSF samples from IE cases were utilised for this experiment (Appendix 8.1.3). All samples were derived from cisterna magna and immediately frozen at -20°C (3 days). CSF samples were defrosted on ice, centrifuged to precipitate cellular debris (see 2.3.1.1, page 79), and the supernatants from each sample were divided into four 50µl aliquots. For each sample, one aliquot was immediately transferred to -80°C storage and used as the control aliquot. The other aliquots were allocated to one of three conditions described in Table 4-1. At the end of treatment the aliquots were returned to -80°C storage.

Storage Scenarios	***In vitro* conditions**
Storage in the fridge overnight	4°C overnight (18 hours)
Carried in the lab coat pocket for 4 hours	37°C for 4 hours
Samples sent by post or left on the bench over the weekend	Room temperature for 48 hours

Table 4-1: Simulated storage conditions from the clinical environment that may affect CSF proteins.

The total protein in each aliquot was determined. Five-µg of protein from each aliquot was prepared for 1-DGE and Western blot analyses.

The number of samples generated for each treatment exceeded the capacity of the gels (maximum sample number per gel=10 plus molecular weight markers), therefore samples were loaded onto paired gels which were run and processed for Western blotting simultaneously. Treated samples were loaded adjacent to its corresponding control. The level of immuno-complexes detected by ECL reagent was quantified as described in 2.3.7.1, page 82. A subtle variation in the intensity of the ECL signal between the two gels was encountered in some experiments most likely attributable to the slight difference in exposure to the ECL solution and the time required towrap the nitrocellulose membrane and place into the X-ray cassette for exposure. Therefore we introduced a correction factor based on the mean value of the control samples of gel 1 and gel 2. This correction value

was applied to all samples. Statistical comparisons between treatment and corresponding control groups were performed by Mann-Whitney *U* test (see 2.6, page 87).

4.3.2.2 The Impact of a Reducing Agent on TTR Dimer Formation

The assessment of TTR stability across the range of selected conditions (Table 4-1) demonstrates a change in the dimer levels following four hours incubation at 37°C (Figure 4-5). To investigate the mechanism that leads to the ratio change between TTR subunits, the effect of TTR dimerisation under reducing conditions was conducted.

Six CSF from IE cases collected from cisterna magna were utilised for this experiment. CSF samples were defrosted on ice, centrifuged to precipitate cellular debris, and the supernatants from each sample were divided into three 50µl aliquots. For each sample, one aliquot was immediately transferred to -80°C storage as control. The other aliquots were incubated at 37°C for four hours with or without the presence of the reducing agent, dithiothreitol (1mM DTT). At the end of treatment the aliquots were returned to -80°C storage.

Five-µg of protein mixed in SDS buffer was denatured and analysed by 1-DGE and Western blot analyses. A reference standard of CSF (randomly chosen from sample bank) was employed at 5µg in each gel to monitor handling variations in this multi-step process (eg., gel-gel variation, ECL exposure). The level of the immuno-complexes detected by ECL reagent was quantified and the density of protein signal was calculated relative to the reference standard and expressed as relative abundance. Statistical comparisons between treatment and corresponding control groups were performed by Kruskal-Wallis ANOVA followed by Dunn's multiple comparison test (see 2.6, page 87).

4.3.3 The Comparative Analyses of Candidate Proteins in DM and Other Neurological Disorders CSF

The selection of DM cases for these experiments was based on the clinical diagnosis and the homozygosity of the mutant allele in *Sod*1 gene (see 3.4.2.1, page 104). Control cases represented by IE, meningoencephalitis (MEN) and chronic intervertebral disc (cIVDD) disease consisted of wild type and heterozygous individuals. Confirmation of diagnoses for these disease categories was determined by routine clinical diagnostics (see 2.2.2, page 76). Samples from patients with acute disease were excluded from analysis (seizure <3

days, acute and rapidly progressive disc disease <48 hours). Any chronic disc cases that were previously diagnosed as DM were also eliminated from further studies.

4.3.3.1 The Comparative Analyses of Candidate Proteins in IE and DM CSF

CSF supernatants were prepared and subsequently desalted using acetone precipitation technique (see 2.3.3, page 80). Five-µg of protein was utilised for 1-DGE and Western blot analyses. A reference CSF standard was employed at 5µg protein in each gel. The level of the immuno-complexes detected by ECL reagent was quantified and the density of protein signals was calculated relative to the reference standard and expressed as relative abundance. Vertical scatter graphs were plotted based on these values and statistically analysed by using Mann-Whitney *U* test.

4.3.3.2 Age-related Influence on the CSF TTR Levels

Human studies on TTR have implied a potential for age-related influence on TTR expression in CSF, although there were inconsistencies between studies (Serot et al. 1997; Chen et al. 2005). Therefore CSF TTR in IE and DM samples were examined for evidence of age-related changes. Correlation analysis for this study was performed using a non-parametric, Spearman's rank correlation coefficient in GraphPad Prism software version 4.0.

4.3.3.3 The Comparative Analysis Candidate Proteins in the CSF of DM and Other Neurological Disorders

Due to insufficient CSF material available for the desalting procedure, the 1-DGE and Western blot analyses for this study were carried out using CSF supernatant (non-precipitated sample).

Five-µg of protein was utilised for each sample. Since the number of CSF samples analysed exceeded the capacity of the gels, samples were loaded over four separate mini gels, which were run and processed for Western blotting simultaneously. A reference standard at two different concentrations, 5µg and 10µg was incorporated in each gel to assess potential handling variations. This consisted of a CSF sample of sufficient volume to generate multiple aliquots that could be incorporated over a series of gel runs. Samples from each neurological condition were loaded alternately, and this had no impact on the

relative signal observed between the different groups. The level of the immuno-complexes detected by ECL reagent was quantified and the levels of TTR calculated relative to the reference standard and expressed as relative abundance. Vertical scatter graphs were plotted based on the values quantified from Western blot and subsequently analysed by using Kruskal-Wallis ANOVA and Dunn's multiple comparison test.

4.4 Results

4.4.1 Validation of Commercial Antibodies in Canine CSF

Figure 4-1 summarises the cross-reactivity of commercial antibodies in canine CSF and brain tissue homogenates. We tested two commercial antibodies for cystatin C, only one antibody (US Biologicals, USA) produced a robust signal in canine CSF. Cystatin C (~13kDa) was detected in mouse and canine brain tissue homogenates although the levels were weaker than the signal observed in canine CSF. Initial Western blot analysis had established that TTR was robust in canine CSF, however TTR could not be detected in either mouse or dog brain homogenates. Two TTR subunits were recognised in Western blot, the dimeric form at 28kDa and monomeric at 13kDa. The antibodies against neuroendocrine 7B2 protein failed to produce a signal in canine CSF, however one antibody produced a weak signal in mouse and dog brain homogenates. The commercial antibodies against VGF (data not shown) have failed to detect this protein in the canine CSF and brain tissue homogenates.

4.4.1.1 Optimisation of TTR Signal for Western blot Analysis

Preliminary Western blot detected two TTR subunits in canine CSF (Figure 4-1B). The optimisation of Western blot on both TTR subunits was conducted in canine CSF to determine optimum protein signal for TTR quantification (Figure 4-2). Gross observation on signal levels at 2.5, 5, 10 and 20µg suggested that 5µg of protein revealed optimum signal for TTR dimer. However, signal saturation was observed in TTR monomer at 5µg, which was not ideal for quantification. Therefore the exposure time of blot on the hyperfilm was reduced to 20 seconds to achieve optimum signal for TTR monomer at 5µg. Although TTR monomer signal was found to be optimum at 2.5µg, signal variation between sample and technical error may be encountered when dealing with very small sample volumes therefore 5µg was used for standard Western blot for TTR.

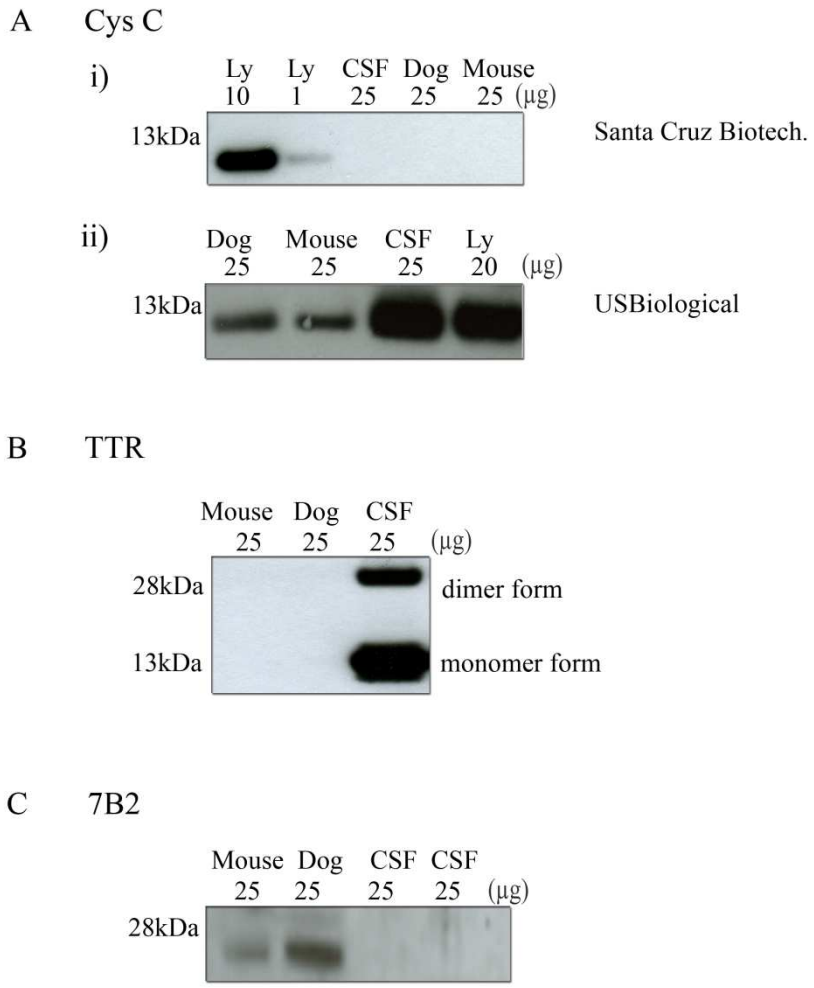

Figure 4-1: The validation of the commercial antibodies in canine CSF and brain tissue homogenates.
A) i) Antibody against cystatin C (Santa Cruz Biotechnology Inc) detects a strong protein signal in lysates (Ly) from cells transfected with cystatin C (Cys C) expressing plasmid but fail to detect cystatin C in canine CSF or brain tissue homogenates. ii) Antibody against cystatin C from US Biological detects the protein in the control cell lysates as well as brain homogenates from mouse and dog at 13kDa. Strong signals in CSF are also observed (13kDa). B) Two bands corresponding to the TTR monomer (13kDa) and dimer (28kDa) are detected in canine CSF. However, there is lack of signal detected in brain tissue homogenates from mouse or dog. C) An antibody against 7B2 (Enzo Life Sciences) detects a protein band at the expected molecular weight (28kDa) in brain tissue homogenates from mouse and dog but fail to detect the protein in canine CSF.

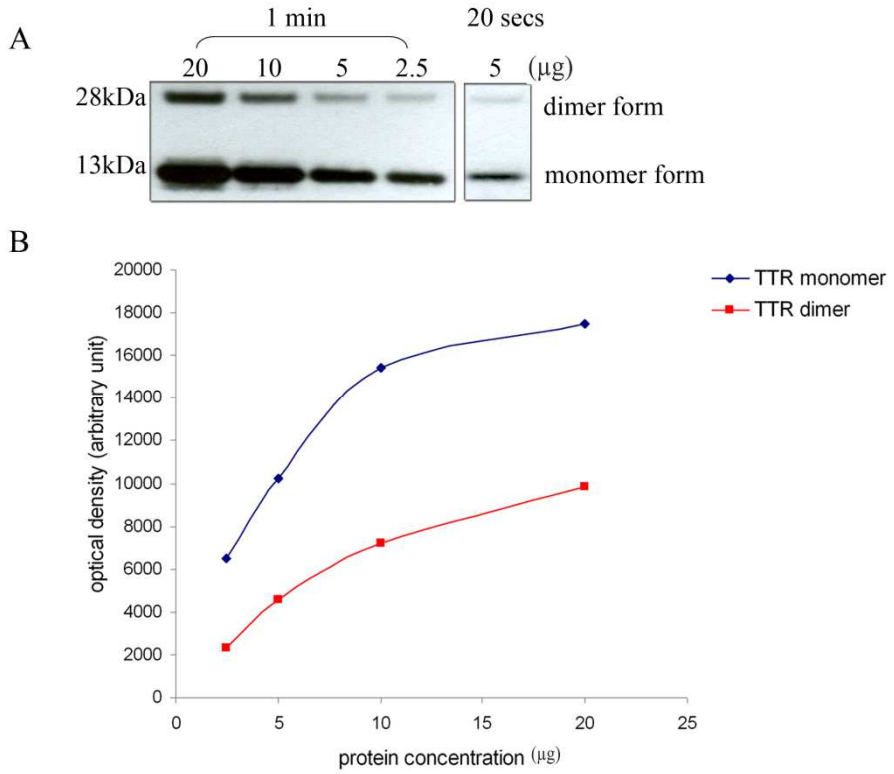

Figure 4-2: The optimisation of TTR signals in Western blot using canine CSF.

A) Western blot demonstrating that 5µg of protein is optimum for TTR dimer with one minute exposure by ECL reaction but not for TTR monomer. Signal of TTR monomer is optimum at 2.5µg, however we predicted that there may be variation observed in TTR dimer signal across canine CSF that could affect protein quantification. Therefore, 5µg was selected for standard Western blot analysis for both TTR subunits. Optimum signal for TTR monomer at 5µg is achieved at 20 seconds exposure to xray film. B) Optical density for both TTR subunits at 2.5, 5, 10, 20µg were quantified and plotted in XY scatter graph. The signal for both TTR subunits are under exponential phase until at 10µg, however the signals start to decrease at 20µg indicating saturation of protein signal.

4.4.2 Pre-Analytical Assessment: Influence of Sample Handling Regimes on the Candidate Protein Levels in Canine CSF

4.4.2.1 Assessment of the Protein Profile by Silver Staining

The gross protein profile for each treatment group was visualised by silver staining and demonstrated a comparable pattern between control and treated samples (Figure 4-3). No sign of degradation was observed in CSF samples. Interestingly, one CSF sample in lane 5 (control) and lane 6 (treated) displayed an additional low molecular weight protein band (>13kDa) that was not apparent in the other samples which may indicate some unique characteristic of this case. An intense band at 62kDa can also be observed in the treated sample in lane 14 in the 4°C group but not in the control aliquot and may be indicative of protein aggregation. This may have been caused by unintentional technical error that perhaps occurred due to the addition of an insufficient amount of denaturing buffer to this sample.

4.4.2.2 Assessment of Cystatin C Levels Following Treatment

The effect of three sample processing temperatures on the level of cystatin C is summarised in Figure 4-4. Cystatin C displayed a stable profile in that the signals were unaffected by storage at 4°C overnight, 37°C for 4 hours or prolonged exposure to ambient room temperature. The means and standard deviations for each control and treatment group are given in Table 4-2.

4.4.2.3 Assessment of TTR Levels Following Treatment

The effect of sample processing temperatures on the level of TTR dimer and monomer in canine CSF is shown in Figure 4-5 and Figure 4-6 respectively. The dimeric TTR levels between treated and control samples were significantly different. An elevation of dimeric levels was demonstrated in the 37°C for 4 hours ($P<0.01$) and room temperature ($P<0.01$) groups. The more abundant monomeric form of TTR was unaffected by all three conditions. Table 4-2 shows the summary of descriptive statistics for control and treatment group.

4.4.2.4 The Impact of a Reducing Agent on TTR Dimer Formation

The elevated level of dimeric TTR following incubation at 37°C was attenuated by pre-treating CSF with the reducing agent DTT. The finding confirms that dimeric TTR formation at 37°C could be inhibited with the inclusion of DTT ($P<0.05$), and suggesting that the association of TTR structure is mediated through disulfide bonds (Figure 4-7). The numerical data for this experiment are presented in Table 4-3.

Protein / Treatment	Cystatin C	TTR dimer	TTR monomer
Control	10760±7057	9431±3288	6732±1800
4°C overnight	11388±6475	10400±3943	8462±2281
Control	10701±4207	9380±5966	11844±5404
37ºC for 4 hours	12373±3394	18792±8320**	13043±4049
Control	10738±4057	9422±4116	11628±2483
RT for 48 hours	8749±3493	15780±4127**	10738±3218

Table 4-2: The cystatin C and TTR optical density values in pre-analytical assessment.

Data presented as mean ± standard deviation and ** corresponds to $P<0.01$.

Treatment	TTR dimer	*P* value
[a]Control	176.2±66.5	ns
[b]37°C – 1mM DTT	225.3±82.1	ns
[c]37°C + 1mM DTT	101.2±52.9	* (b vs. c)

Table 4-3: The data for control and treated groups in TTR dimerisation experiment.

Data displayed as mean ± standard deviation, values calculated in relative to reference standard. * is equivalent to $P<0.05$ and ns is not significant.

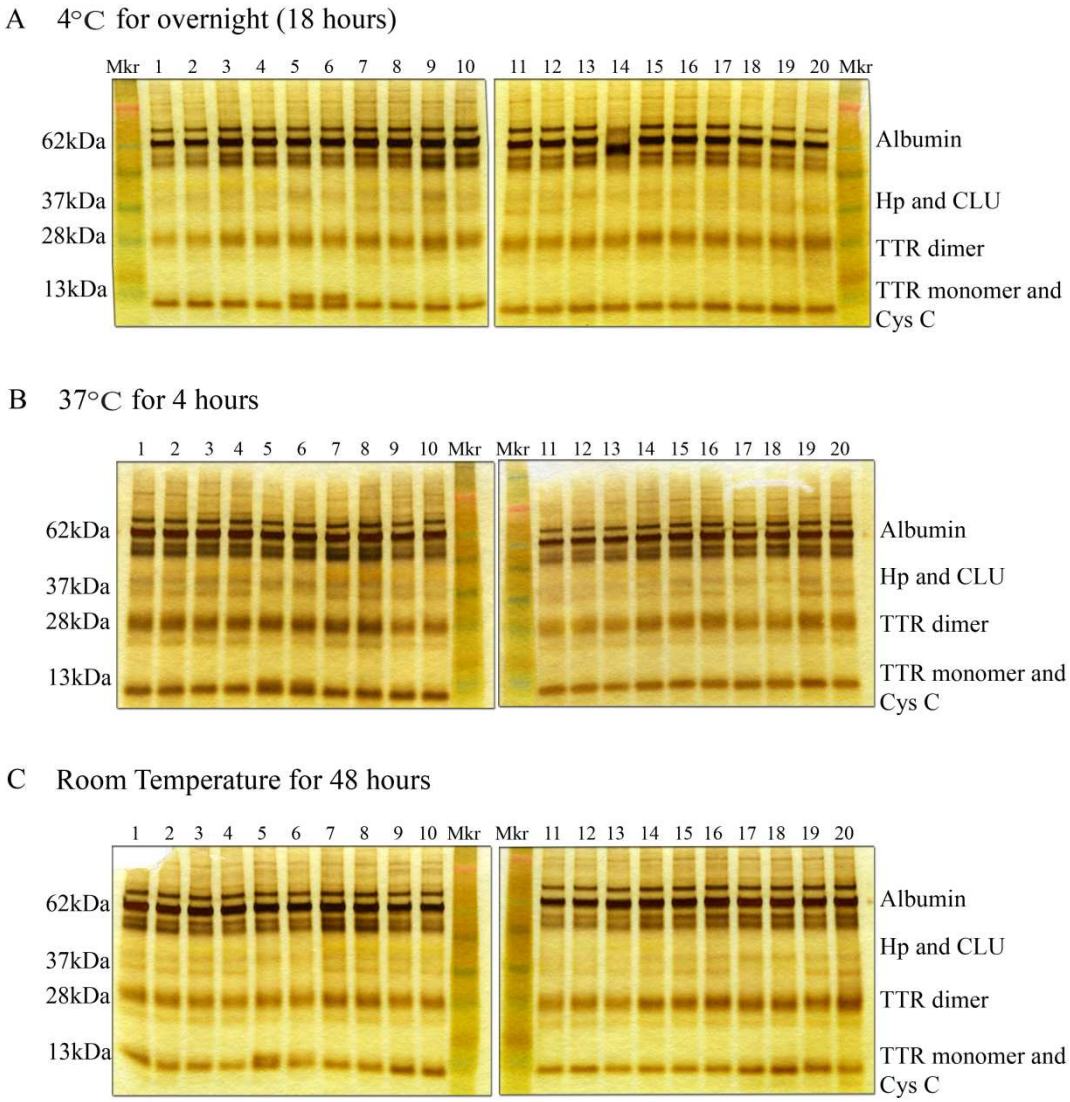

Figure 4-3: Assessment of the influence of sample handling regime on protein profile by silver staining. A)-C) Aliquots of treated (lane with even numbers) and untreated (lane with odd numbers) CSF were resolved on a 1-DGE with inclusion of protein marker (Mkr). The protein profiles were assessed by silver staining and are comparable across the different samples analysed. There is no gross impact on the protein profiles as a consequence of the three different treatment regimes. One CSF sample in lane 5 (control) and 6 (treated) displays an additional protein band (~13kDa) which is consistent in all stained gels. In A) lane 14 demonstrates an intense protein band at 62kDa, however this is not found in other gels. The specific proteins in the silver-stained gels were estimated by their corresponding molecular weight size. Hp – haptoglobin; CLU – clusterin.

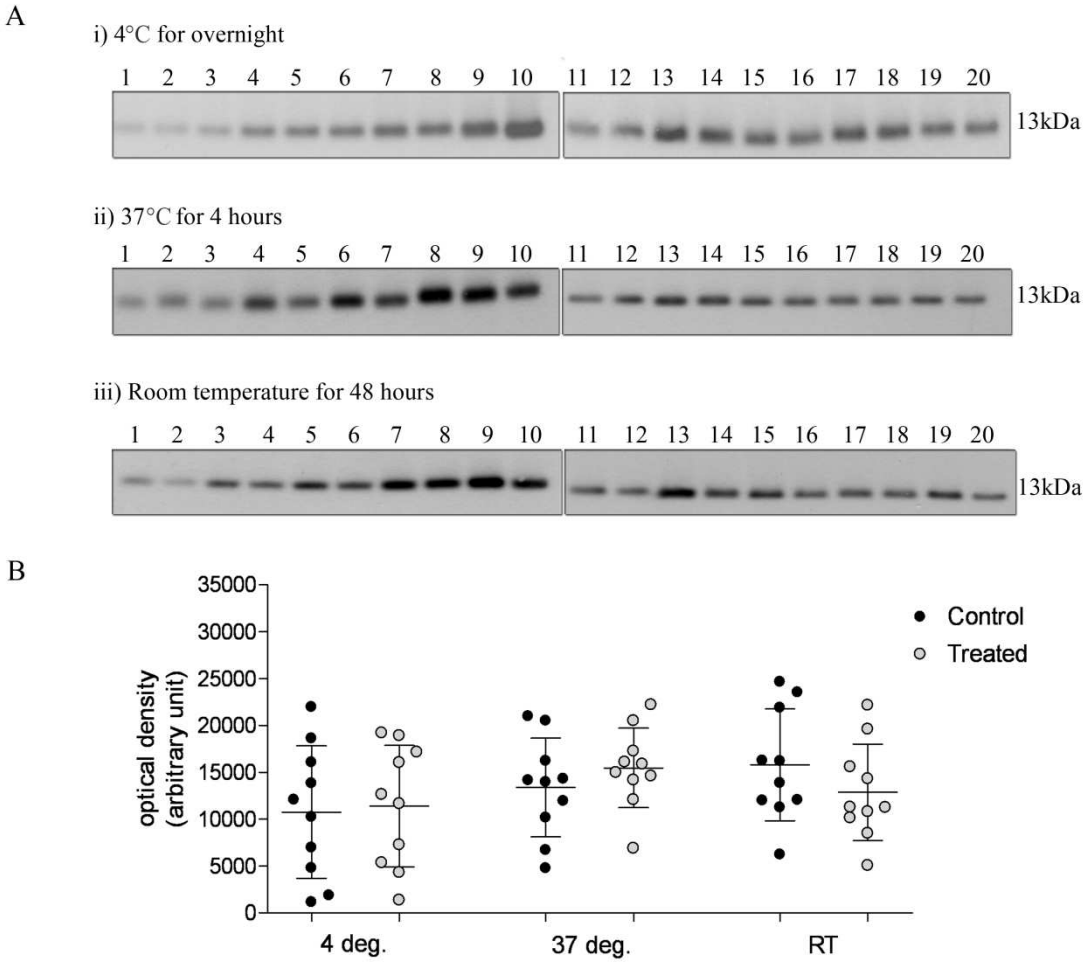

Figure 4-4: The influence of three potential sample handling regimes on cystatin C stability.

A) Cystatin C Western blot signals (*N*=10) for i) 4°C overnight, ii) 37°C, and iii) room temperature B) The protein optical densities were quantified, corrected for gel handling variations, expressed as arbitrary unit, and plotted in vertical scatter graph. Cystatin C protein levels are not altered under all conditions examined. Data presented as mean ± standard deviation.

Lane with odd numbers - control CSF; Lane with even numbers - treated CSF

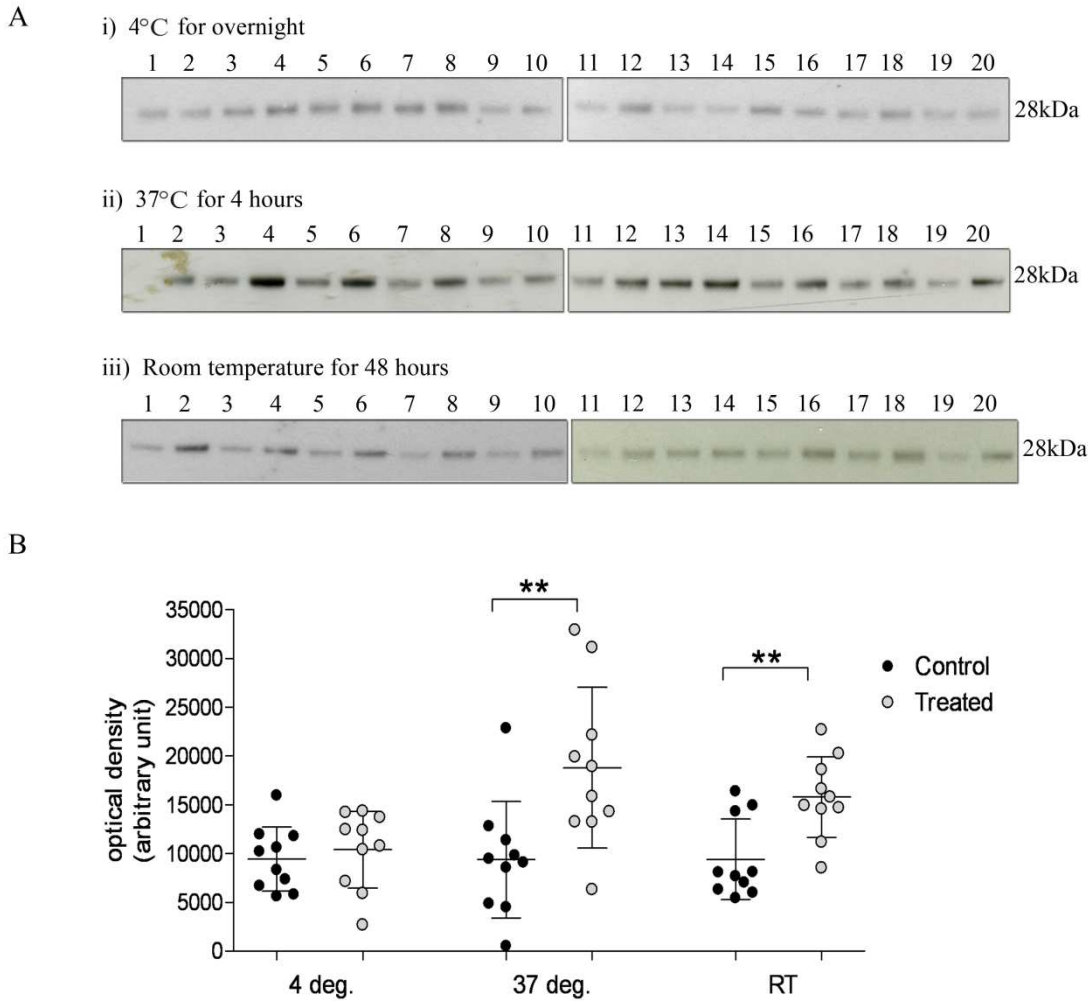

Figure 4-5: The influence of three potential sample handling regimes on TTR dimer stability.

A) The Western blot signals (*N*=10) for TTR dimer for i) 4°C overnight, ii) 37°C, and iii) room temperature. B) The protein optical densities were quantified, corrected for gel handling variations, expressed as arbitrary unit and plotted in vertical scatter plot. Statistical analysis demonstrated that CSF exposure to 37°C for 4 hours and at room temperature for 48 hours result in a significant elevation in the TTR dimer complex but is not influenced by 4°C exposure. Data presented expressed as mean ± standard deviation and ** corresponds to $P<0.01$.

Lane with odd numbers - control CSF; Lane with even numbers - treated CSF

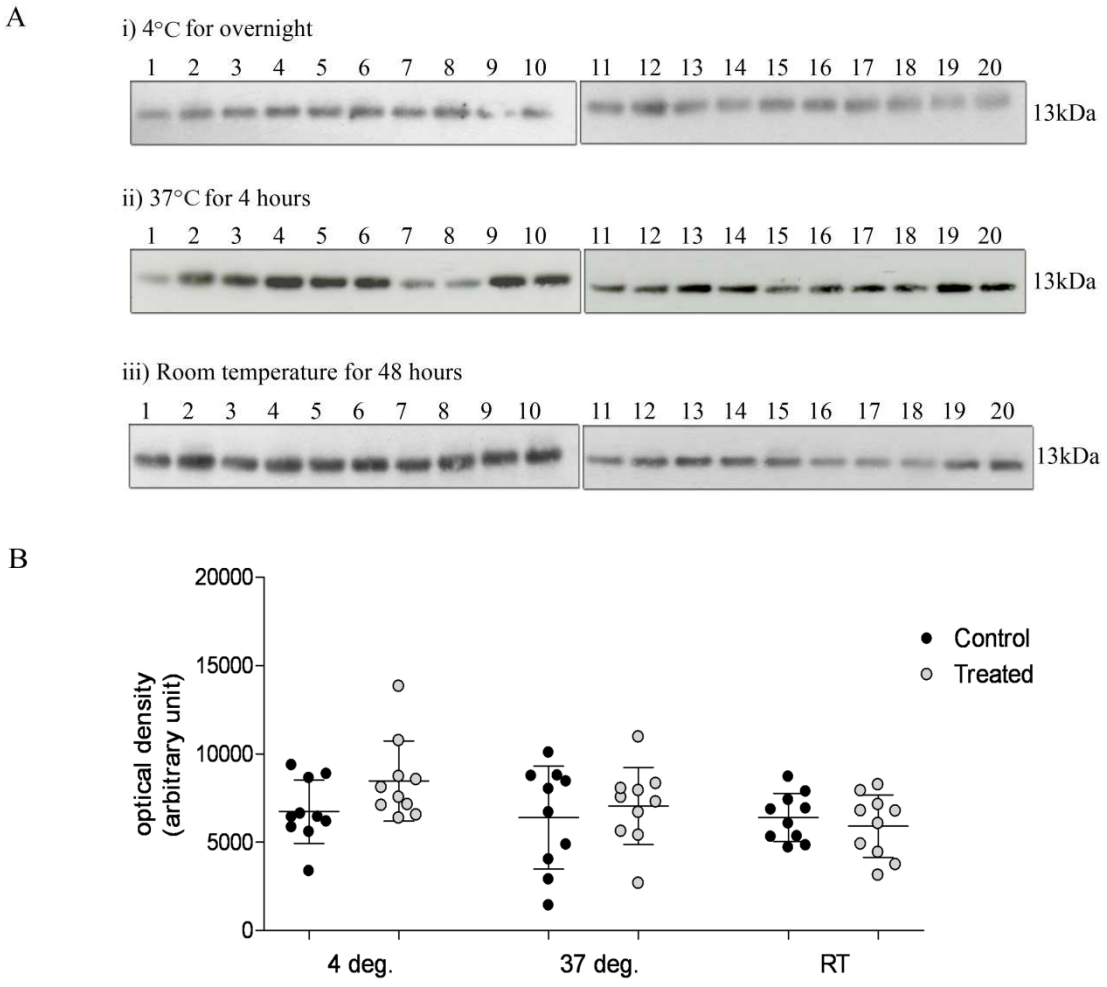

Figure 4-6: The influence of three potential sample handling regimes on TTR monomer stability.

A) The Western blot signals (*N*=10) for TTR monomer for i) 4°C overnight, ii) 37°C, and iii) room temperature. B) The protein optical densities were quantified, corrected, expressed as arbitrary unit, and plotted using vertical scatter plot. TTR monomer levels are not altered under all conditions examined. Data presented as mean ± standard deviation.

Lane with odd numbers - control CSF; Lane with even numbers - treated CSF

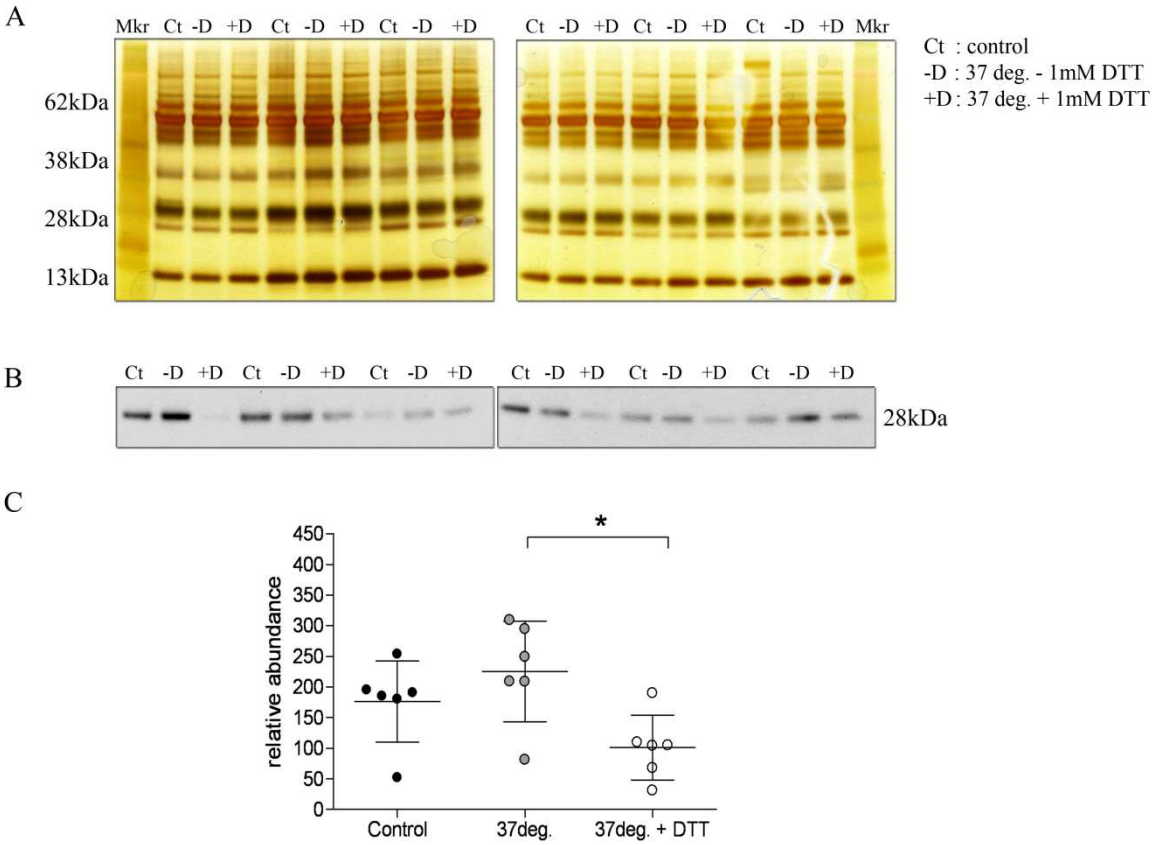

Figure 4-7: The reducing agent DTT blocks the TTR dimer formation at 37°C.

A) CSF samples were incubated at 37°C for 4 hours with or without DTT (1mM) and subsequently compared with immediately denatured controls (*N*=6 for each group). The protein profiles of controls, treated aliquots with or without DTT were assessed by silver staining. Gross observation on silver stained gels indicates no sign of protein degradation. B) The protein densities from Western blot were quantified and the vertical scatter graph was plotted based on the signal levels detected by ECL. Statistical analysis demonstrated that the presence of DTT in samples incubated at 37°C resulted in a significant reduction in TTR dimer levels compared to without DTT samples treated at 37°C. Data presented as mean ± standard deviation and * corresponds to $P<0.05$.

4.4.3 The Comparative Analyses of Candidate Proteins in DM and Other Neurological Disorders CSF

Prior to Western blot analysis, all CSF aliquots used in gel-based analysis were assessed for signs of protein degradation (data not shown).

All samples marked "X" were excluded in statistical analyses based on the selection criteria summarised in 4.3.3, page 116. CSF sample collected from lumbar cistern was marked as "L" and the protein value was indicated by a pink-coloured dot in the vertical scatter plot. The complete list of dogs included in each analysis is given in Appendix 8.1.1.

4.4.3.1 Comparative Analysis of Cystatin C in IE and DM CSF

The relative concentrations of cystatin C in IE and DM CSF demonstrated no significant difference between groups (Figure 4-8). The means and degree of variations for this analysis are given in Table 4-4.

4.4.3.2 Comparative Analysis of TTR in IE and DM CSF

The relative concentrations of TTR dimer (Figure 4-9) and monomer (Figure 4-10) in DM were significantly reduced compared to IE CSF (TTR dimer, $P<0.05$, TTR monomer, $P<0.01$). The summary of means and standard deviations for this experiment are presented in Table 4-4.

4.4.3.3 Age-related Influence on the CSF TTR Levels

CSF TTR levels in both of IE and DM groups were examined for age-related changes. Correlation analysis revealed a weak negative relationship between TTR subunits levels and age, but the relationships were not statistically significant (Figure 4-11).

4.4.3.4 Comparative Analysis of TTR in CSF from Various Neurological Disorders

The comparative analysis of TTR dimer and monomer levels across the neurological conditions is shown in Figure 4-12 and Figure 4-13 respectively. Statistical analysis in both TTR subunits levels demonstrated no significant difference between disease groups. The numerical findings for this investigation are shown in Table 4-5.

Protein / Group	Cystatin C	TTR dimer	TTR monomer
IE	86.8±62.1	96.9±52.5	149.2±43.4
DM	107.6±50.8	48.2±12.5*	77.7±24.0**

Table 4-4: The cystatin C and TTR levels in IE and DM CSF.

The data presented as mean ± standard deviation, values calculated in relative to reference standard. * is $P<0.05$ and ** is $P<0.01$.

Protein / Group	TTR dimer	TTR monomer
[a]IE	77.1±44.7	117.1±49.5
[b]DM	84.3±35.6	127.7±25.5
[c]MEN	89.2±27.6	123.7±41.2
[d]cIVDD	91.0±61.1	151.3±71.7

Table 4-5: The CSF TTR dimer and monomer values in various neurological disorders.

Data displayed as mean ± standard deviation, values calculated in relative to reference standard.

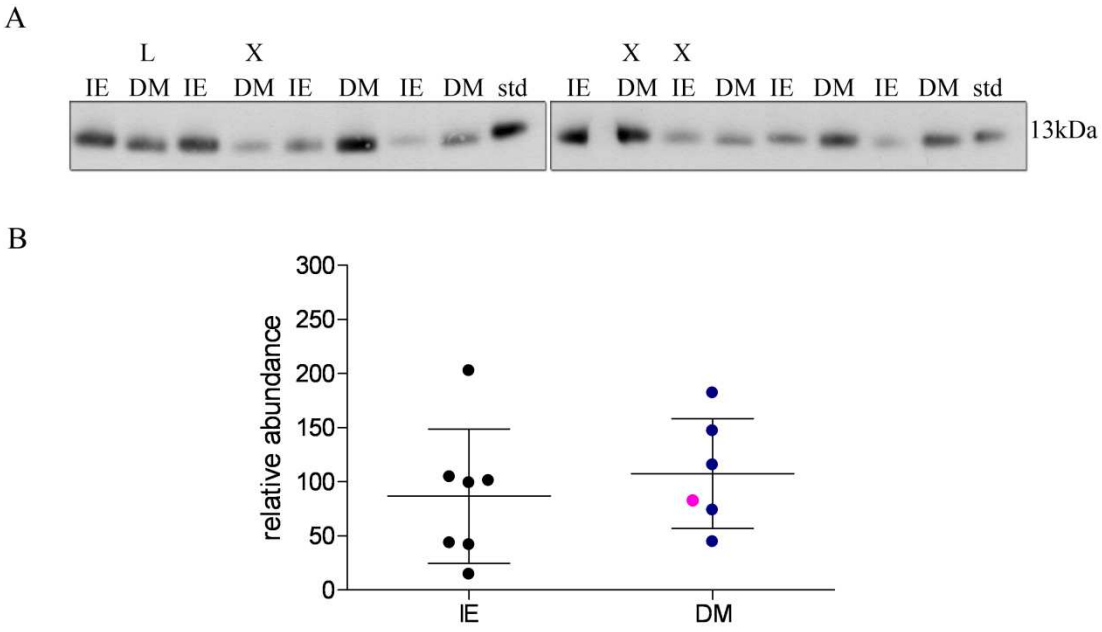

Figure 4-8: The comparative analysis of cystatin C in IE and DM CSF.

A) The cystatin C signals obtained from Western blot analysis in IE (*N*=7) and DM (*N*=6) cases. B) Vertical scatter graph was plotted to show the data distribution. Statistical analysis demonstrated no significant difference in cystatin C levels between IE and DM groups. Samples marked "X" were excluded from statistical analysis. Sample marked "L" was collected from lumbar CSF and the protein value from this sample is represented as pink-coloured dot in the vertical scatter graph. Data presented as mean ± standard deviation.

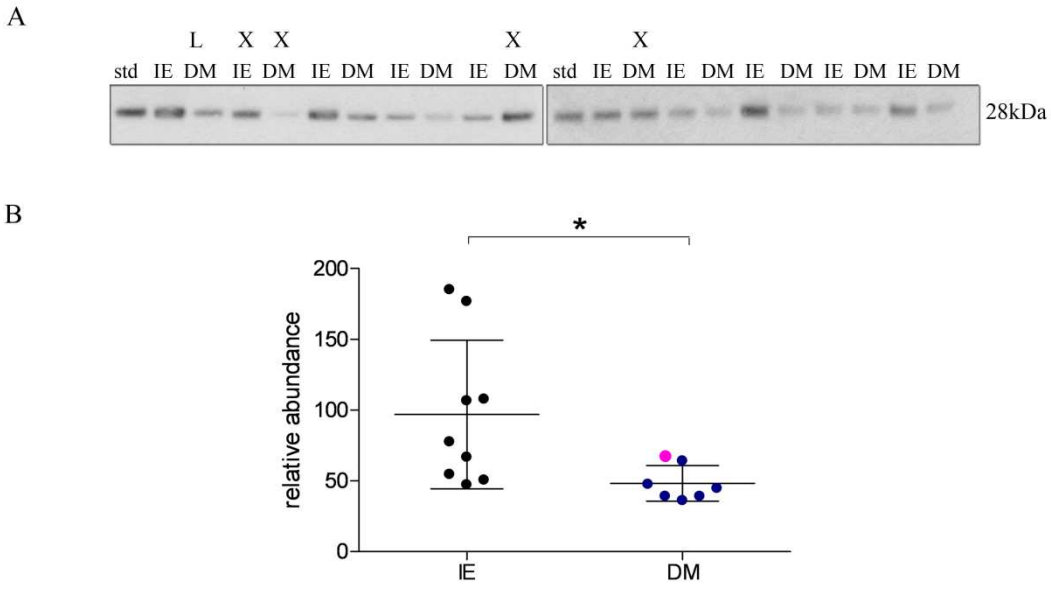

Figure 4-9: The comparative analysis of TTR dimer in IE and DM CSF

A) TTR dimer signals obtained from Western blot analysis. B) Vertical scatter graph was plotted to show the data distribution. Statistical analysis revealed significant reductions in TTR dimer levels between IE (*N*=9) and DM (*N*=7) groups. Samples marked "X" were excluded from statistical analysis. Sample marked "L" was collected from lumbar CSF and the protein value from this sample is represented as pink-coloured dot in the vertical scatter graph. Data presented as mean ± standard deviation and * corresponds to $P<0.05$.

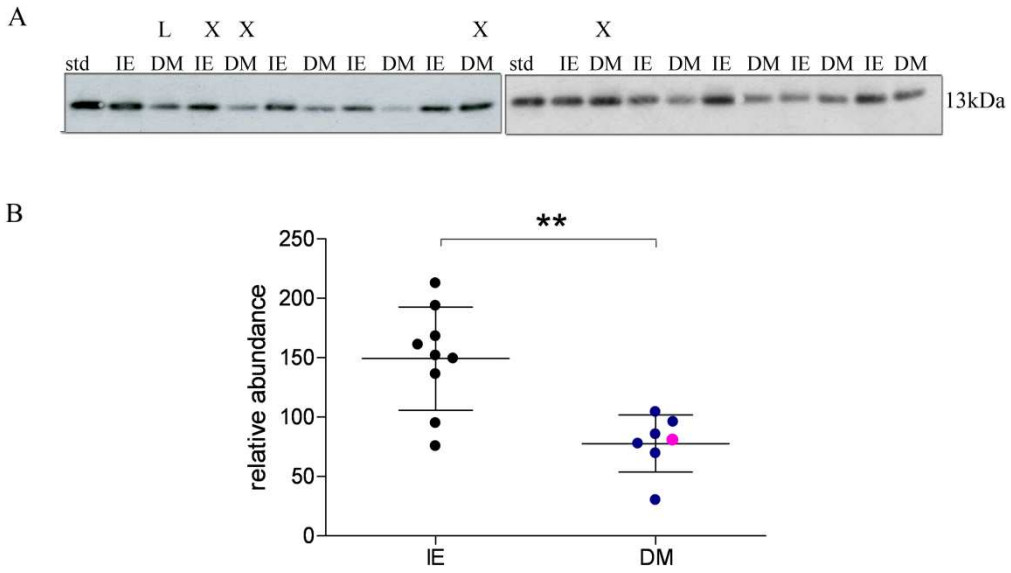

Figure 4-10: The comparative analysis of TTR monomer in IE and DM CSF.

A) TTR monomer signals obtained from Western blot analysis. B) Vertical scatter graph was plotted to show the data distribution. Statistical analysis demonstrated significant reductions of TTR monomer levels between IE (*N*=9) and DM (*N*=7) groups. Samples marked "X" were excluded from statistical analysis. Sample marked "L" was collected from lumbar CSF and the protein value from this sample is represented as pink-coloured dot in the vertical scatter graph. Data presented as mean ± standard deviation and ** corresponds to $P<0.01$.

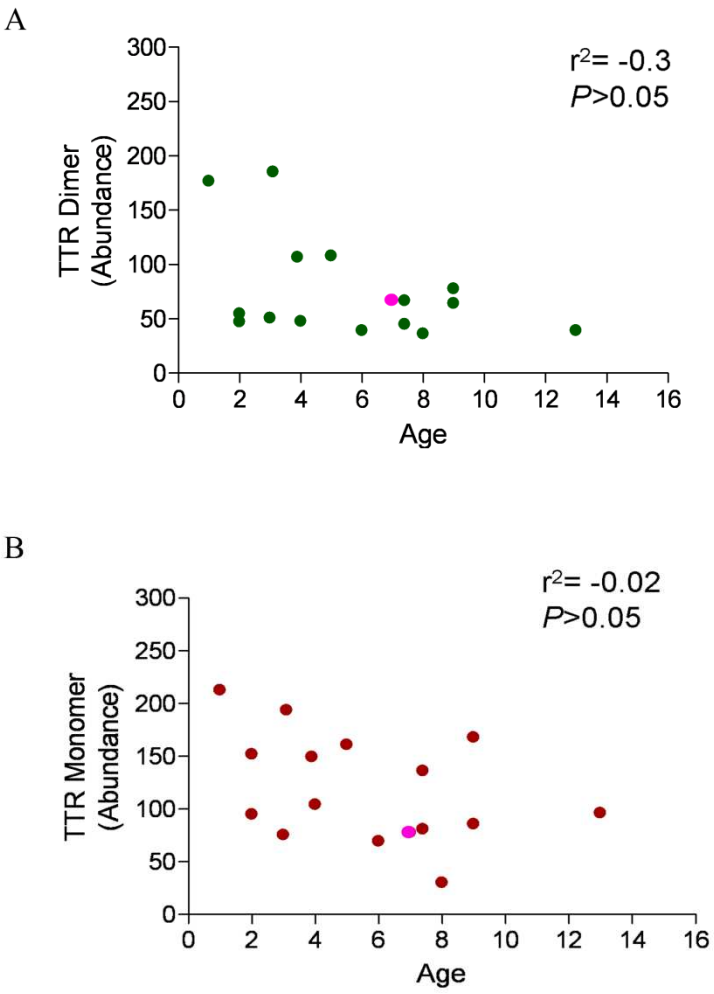

Figure 4-11: The correlation analysis of TTR subunits levels versus age.

A) Correlation analysis of TTR dimer versus age. B) Correlation analysis of TTR monomer versus age. There are no significant relationships between the TTR subunits levels and the age of the cases analysed. Protein values indicated by pink-coloured dots represent lumbar CSF.

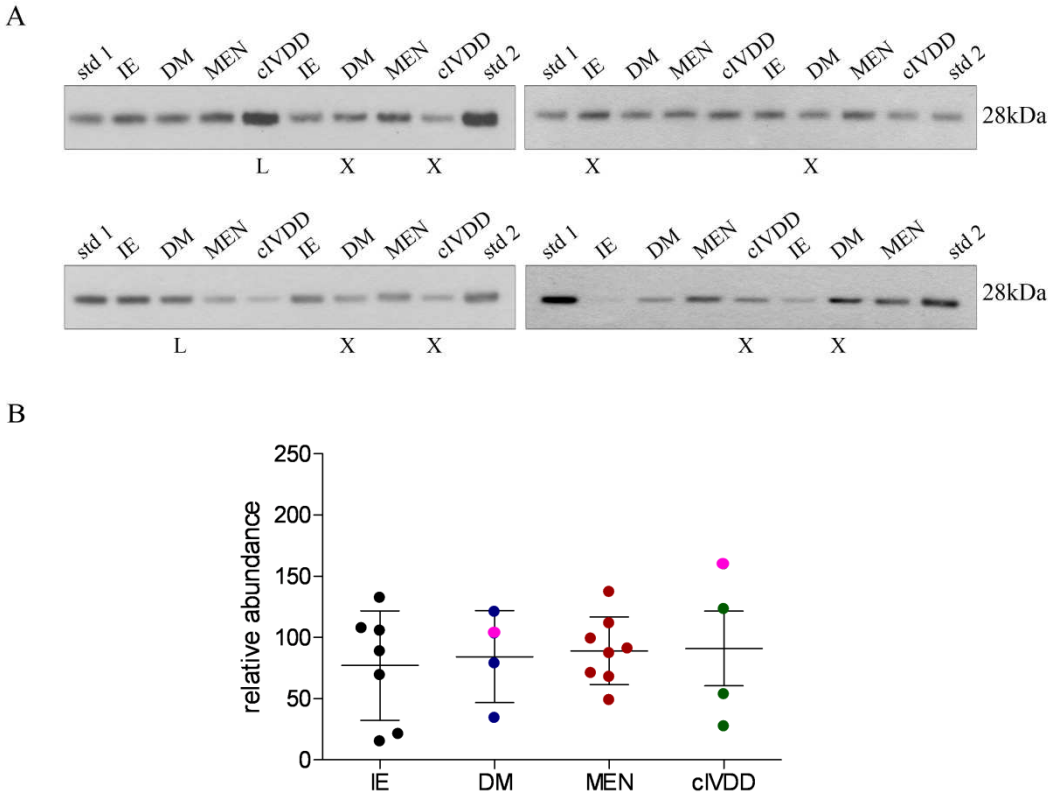

Figure 4-12: The comparative analysis of TTR dimer in various neurological disorders CSF.

A) TTR dimer signals obtained from Western blot analysis. B) Signals were quantified and values were plotted in vertical scatter plot. Statistical comparison between four disease groups; IE (*N*=7), DM (*N*=4), MEN (*N*=8), and cIVDD (*N*=4) demonstrate no significant difference. Samples marked "X" were excluded from statistical analysis. Sample marked "L" was collected from lumbar CSF and the protein value from this sample is represented as pink-coloured dots in the vertical scatter graph. Data presented as mean ± standard deviation.

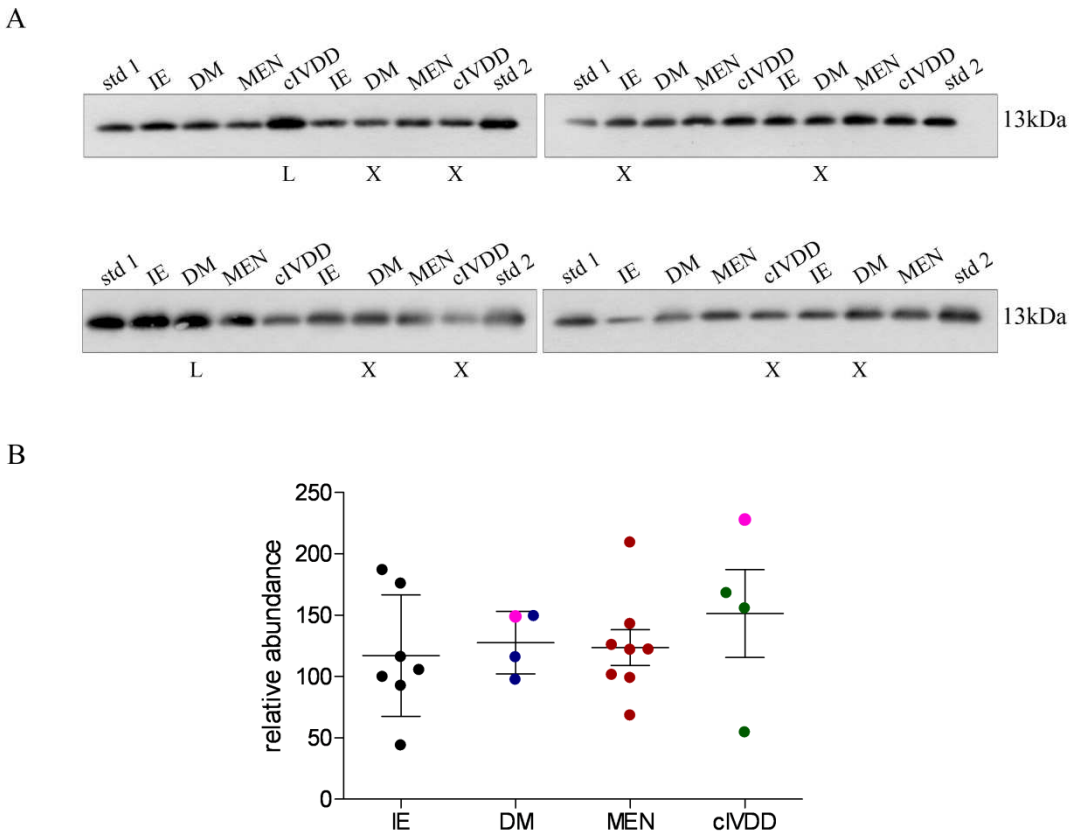

Figure 4-13: The comparative analysis of TTR monomer in various neurological disorders CSF.

A) TTR monomer signals obtained from Western blot analysis. B) Signals were quantified and values were plotted in vertical scatter plot. Statistical comparison between four disease groups; IE (*N*=7), DM (*N*=4), MEN (*N*=8), and cIVDD (*N*=4) demonstrate no significant difference. Samples marked "X" were excluded from statistical analysis. Sample marked "L" was collected from lumbar CSF and the protein value from this sample is represented as pink-coloured dots in the vertical scatter graph. Data presented as mean ± standard deviation.

4.5 Discussion

In this chapter we evaluated the putative ALS biomarkers in DM CSF to determine their potential utility as DM biomarkers. The first step in this study was to validate the commercial antibodies against ALS-associated biomarkers in canine CSF. The preliminary findings of Western blot demonstrated that some antibodies detected the appropriate proteins in canine CSF and were therefore suitable for further study. The discrepancy in the cross-reactivity of these antibodies in canine CSF is expected since most of the commercial antibodies are likely to be tested on murine or human biological samples and to a lesser extent on other species. Four candidate proteins of ALS were evaluated by Western blot, however only cystatin C and TTR have displayed robust signals in canine CSF. Characterisation of 7B2 and VGF proteins in canine CSF were unsuccessful even after testing a range of antibodies obtained from different sources. 7B2 protein was detected in both mouse and dog brain tissue homogenates, which may reflect its high concentrations in the brain tissue compared to CSF as observed in previous studies (Iguchi et al. 1985; Iguchi et al. 1987). Interrogation of genomic sequences in public database (PubMed, NCBI, USA) revealed human 7B2 (Accession: NP_003020.3) to be 92% homologous with canine 7B2 protein (Accession: XM_535423). The failure to identify 7B2 protein in canine CSF could be due to low 7B2 abundance in canine CSF but not due to epitope specificity. Assessment of VGF to predict species divergence between human and dog has been limited by a paucity of published data. The lack of signal detection for VGF by Western blot in canine CSF may be contributed by the lack of specificity of commercial antibodies.

4.5.1 Cystatin C

Preliminary Western blot demonstrated that cystatin C was robust in canine CSF with an estimated molecular weight around 13kDa. The unsuccessful identification of cystatin C at the initial stage using a monoclonal antibody (Enzo Life Sciences, UK) might be a consequence of the lack of a recognisable epitope. It is common for monoclonal antibodies to be able to detect only one epitope in an antigen, which reduces its cross-reactivity and is an issue to be considered when extrapolating observations between species. Therefore polyclonal antibodies were used for validation of cystatin C and other proteins in canine CSF.

Investigation of protein biomarkers may be affected by various pre-analytical factors that can arise from the clinical and laboratory environments (Ferguson et al. 2007; Pieragostinoa et al. 2010). In this study the geographical distance between the small animal hospital and the laboratory could potentially compromise the reliability of samples subjected to biomarker analyses. Therefore the assessment of the impact of three potential sample handling practices on the cystatin C levels in canine CSF was conducted prior to comparative analysis. Our study has demonstrated that cystatin C in canine CSF was not altered by prolonged CSF storage at 4°C or 37°C or even with long exposure of CSF at ambient temperature. There is no report available on the effect of short term storage of CSF on cystatin C, however cystatin C has been reported to be stable when CSF was stored at -20°C for three months (Carrette et al. 2005). The prolonged storage of CSF beyond the three months period however demonstrated a truncated cystatin C form (Carrette et al. 2005; Boccio et al. 2006). Another study on urinary cystatin C in human has indicated that cystatin C was clearly stable at -20°C and 4°C for 7 days, and at 20°C for 48 hours (Herget-Rosenthal et al. 2004). However urinary cystatin C appeared to be significantly reduced after 72 hours at 20°C. The same study also shown that cystatin C was not influenced by three freezing and thawing cycles (Herget-Rosenthal et al. 2004). Although cystatin C is shown to be stable across the storage conditions of canine CSF, evidence in the literature does highlight the importance of the optimal storage at -80°C to avoid complication in cystatin C biomarker analysis.

In ALS, a significant reduction of cystatin C levels is reported in CSF of ALS patients and is recognised as one of the most promising biomarkers of ALS (Ranganathan et al. 2005; Pasinetti et al. 2006; Ryberg et al. 2010; Wilson et al. 2010). The down-regulation of cystatin C in ALS CSF also has been correlated with disease duration in patients with spinal onset of ALS (Ryberg et al. 2010). The causative mechanism leading to the reduction of cystatin C in ALS has not been defined, however evidence has shown that reduction of this protein may enhance protein degradation and prevent cellular repair (Nakanishi, 2003; Olsson et al. 2004). However comparative analysis of cystatin C levels between IE and DM groups in this study has demonstrated no significant difference and therefore it is not a strong contender as a DM biomarker.

4.5.2 TTR

TTR in CSF exists predominantly in its monomeric form (13.8kDa) (Dickson et al. 1986; Schreiber et al. 1990; Zheng et al. 1999), although a higher molecular weight of TTR

known as the dimeric form can also be found (Chen et al. 2005; Foss et al. 2005). TTR primarily originates from the liver, however in CSF TTR is present in high concentrations and is exclusively produced by the choroid plexus (Ingenbleek and Young, 1994). In CNS tissue TTR is expressed in low concentrations and is transported to the CNS parenchyma from blood and CSF (Chanoine et al. 1992). The preliminary Western blot revealed both TTR monomer and dimer subunits in canine CSF at 13kDa and 28kDa respectively. We also confirmed that TTR monomer was found in greater concentration than TTR dimer in canine CSF with a monomer to dimer ratio of 5:1. The high concentration of TTR monomer in this study is consistent with previous TTR studies in dogs ((Forterre et al. 2006; Piechotta et al. 2012), rat (Schreiber et al. 1990) and sheep (Marchi et al. 2003), although slight variation in terms of molecular weight may be observed depending on the species and the type of antibody used in the laboratory (Chen et al. 2005). TTR was not detected in mouse or dog brain homogenates, however, this could be due to the low TTR expression in CNS tissue (Stein and Johnson, 2002; Ranganathan et al. 2005).

The stability of both TTR subunits was assessed. We initially hypothesised that prolonged storage of CSF at high temperatures (4°C, 37°C, room temperature) may cause a significant reduction of TTR due to protein degradation. However an unexpected increment pattern was observed in the level of dimeric TTR in canine CSF at 37°C and at room temperature. TTR monomer levels were unaffected by these conditions however, due to the relativity high concentration of monomeric TTR in canine CSF, we speculated that the predicted concomitant reduction in the monomeric pool may not be obvious. This is shown by the minor reduction in monomeric TTR that would imply a spontaneous dimerisation event. To our knowledge there are no current reports on the effect of short term storage on TTR in CSF, although it has been reported that serum TTR was significantly reduced following sample storage at -20°C for three months (Pieragostinoa et al. 2010). A report on CSF TTR has indicated that this protein is sensitive to repeated freeze-thawing cycles (5-10 times) (Rosenling et al. 2009).

The elevation of TTR dimeric levels following 37°C and room temperature treatments may be a consequence of *de novo* synthesis although this is unlikely as all samples were taken from the same route and condition. Therefore we speculated that under these conditions there is an association between monomeric subunits that form into dimeric complexes and that this may occur due to disulphide bridge formation between monomers (Redondo et al. 2000; Foss et al. 2005). 1-DGE and Western blot analysis of treated CSF at 37°C in the

presence of 1mM DTT (a disulphide reducing agent) resulted in a significant reduction (50%) of TTR dimer signals compared non-DTT samples confirming that disulphide bonds are tethering the monomeric subunits together. In this experiment we have indicated that the increased TTR dimerisation is induced by monomer interactions that subsequently lead to the TTR dimer elevation in CSF under 37°C and at room temperature conditions.

Significant reductions of TTR dimer (49%) and monomer (52%) were detected in DM CSF. These findings are consistent the CSF TTR levels observed in the previous ALS studies (Ranganathan et al. 2005; Kolarcik et al. 2007; Ryberg et al. 2010). Therefore, it is possible that the reduction of TTR observed in DM CSF may reflect common pathways with ALS. The mechanism of TTR reduction in CSF has not been fully elucidated however recent studies have identified alterations in post-translational modification of TTR in ALS CSF that may contribute to the significant reduction of TTR levels (Kolarcik et al. 2007; Ryberg et al. 2010). Besides its function as the carrier of thyroid hormones, TTR can act as a chaperone that has the ability to bind or sequester abnormal proteins such as Aβ peptide (Tsuzuki et al. 2000). In addition, TTR also regulates the retinoid signalling pathway, which is involved in neuroplasticity and regeneration (Mey and McCaffery, 2004). Therefore a reduction of TTR levels may lead to inadequate sequestration of abnormal proteins, impairment of signalling and a decrease in the regenerative capacity during CNS tissue injury, which eventually contributes to lesion development in ALS (Kolarcik et al. 2007).

Speculations that TTR levels in CSF may reduce with age was proposed based on the morphological and functional changes of choroid plexus in relation to the ageing process (Serot et al. 2003). A reduction in choroid plexus function corresponding to increased age has been reported (Preston, 2001). In addition, a study of TTR in old sheep demonstrated a significant reduction of CSF TTR levels (Chen et al. 2005), while previous studies of TTR in human CSF have shown inconsistent results (Zheng et al. 2001; Kleine et al. 1993; Serot et al. 1997; Kunicki et al. 1998). Since DM is characterised as a late onset condition it is imperative to determine whether the age factor has an impact on the TTR observation in DM CSF. The correlation analysis of both TTR subunit levels revealed no significant relationship with age in the cases analysed, therefore we are confident that the significant differences of the TTR levels between IE and DM groups is disease-specific.

Further comparative analysis of CSF TTR in various neurological disorders revealed no significant findings, which is inconsistent with the preliminary analysis of TTR in IE and

DM CSF. This comparative analysis of TTR across selected neurological conditions was conducted using CSF supernatant instead of precipitated CSF, thus, it is conceivable that differences in sample preparation could affect the protein profile detected by Western blot. Acetone precipitation is a recommended desalting protocol that is efficient in the removal of high salt and albumin contents that may reduce the efficiency of separation or migration of protein during gel electrophoresis (Yuan et al. 2002). This desalting technique was initially optimised in this study for advance protein separation protocol (2-DGE), however this technique was abandoned at the later stage of the project due to the high requirement of sample volume and failure in optimising 2-DGE protocol in canine CSF (Appendix 8.5.1). Series of 1-DGE analyses on CSF supernatants have demonstrated clean and robust protein profiles in polyacrylamide gel and Western blot, implying that desalting procedure may not be critical in 1-DGE protein separation. There were also no differences observed in the molecular weight size of both TTR subunits.

We proposed that TTR is a potential candidate protein for DM biomarker, however further evaluation of TTR in a large scale DM population is required to determine the specificity of this protein as a clinical biomarker for DM. This objective will be accomplished once the clinical material becomes available in future.

5 The Characterisation of Novel CSF Biomarkers in DM

5.1 Background

The search for novel protein biomarkers for human diseases is being actively pursued by researchers from many disciplines since biomarkers have the potential to inform on diagnosis, disease progression and underlying pathological mechanisms (Ryberg and Bowser, 2008; Perrin et al. 2011). Collectively, this information may enhance the likelihood of the development of therapeutic agents where their effectiveness may also be monitored with appropriate biomarker assessment (Abdi et al. 2006; Yoon and Sin, 2011). Proteomic studies have generated complementary datasets to genomics that could be used simultaneously to provide further understanding of biological systems (Tyers and Mann, 2003). In this project the identification of novel protein biomarkers in DM CSF using a proteomic approach will not only provide additional information on the DM pathogenesis but may also complement the *Sod*1 genotyping to achieve specific diagnosis in the clinic. Two strategies; 1-DGE and 2-DGE techniques were initially adopted and progressed in parallel, however initial assessment of 2-DGE system in canine CSF revealed unsatisfactory protein separation (see Appendix 8.5.1). Preliminary results of 1-DGE demonstrated robust data therefore a combination of 1-DGE and MALDI-TOF MS was employed for novel biomarker identification.

5.2 Aims

In this chapter, we aimed to identify and characterise the novel biomarkers in DM CSF through a gel-based technique, 1-DGE coupled with MALDI-TOF MS. The specific aims of work presented in this chapter were to:

1. Identify potential biomarkers in DM CSF through high throughput MALDI-TOF MS.

2. Validate the cross-reactivity of commercial antibodies against novel candidate proteins in canine CSF.

3. Assess the influence of potential sample handling practices on the candidate protein levels in canine CSF.

4. Perform comparative evaluations of the candidate protein levels in DM and other neurological disorders CSF.

5.3 Materials and Methods

5.3.1 Identification of Potential Biomarkers in DM CSF through MALDI-TOF MS

CSF samples from IE and DM homozygotes were defrosted on ice and centrifuged to remove cellular debris (see 2.3.1.1, page 79). CSF supernatants were desalted using acetone precipitation technique (see 2.3.3, page 80) and prepared and analysed for 1-DGE (see 2.3.5, page 80). The gel was stained with Coomassie blue (see 2.3.6, page 81) and the protein profiles were assessed. The band of interest was excised, transferred to 1.5ml eppendorf tube and transported at room temperature to the proteomic facility in University of Glasgow (under directorship of Dr. Richard Burchmore). The gel was cut into several pieces and subjected to trypsin enzyme digestion for MALDI-TOF MS analysis (see 2.3.8, page 83).

5.3.2 Validation of Commercial Antibodies in Canine CSF

Validation of commercial antibodies against novel candidate proteins was performed using CSF supernatants and compared with dog and mouse brain tissue homogenates using Western blots as described in 4.3.1, page 114. The details of commercial antibodies for the proteins identified by MS (clusterin and haptoglobin) is summarised in Table 2-1.

5.3.3 Pre-Analytical Assessment: Influence of Sample Handling Regimes on Candidate Protein Levels in Canine CSF

The candidate proteins identified in MALDI-TOF MS were subjected to three potential CSF sample handling regimes modelled in the laboratory; 4°C for 18 hours, 37°C for four hours and room temperature for 48 hours (see 4.3.2, page 115). Five-µg of protein from CSF supernatant was utilised for this assessment and experiments were executed as detailed in 4.3.2.1, page 115. Following 1-DGE (see 2.3.5, page 80) Western blot analyses (see 2.3.7, page 81) the protein signal was quantified (see 2.3.7.1, page 82), corrected for gel handling variations and plotted using vertical scatter plot in arbitrary units. Statistical

comparison between treatments and corresponding controls was achieved using a non-parametric, Mann-Whitney *U* test (see 2.6, page 87).

5.3.4 The Comparative Analyses of Candidate Proteins in DM and Other Neurological Disorders CSF

The case selection for each disease group; IE, DM, MEN and cIVDD was achieved based on the clinical diagnosis and the *Sod*1 genotyping as detailed in 4.3.3, page 116.

5.3.4.1 Investigations of Candidate Proteins Levels in IE and DM CSF

Five-µg of protein from CSF supernatants were utilised for 1-DGE and Western blot analyses. A reference standard was employed at 5µg in each gel. The level of immune-complexes was quantified and the relative concentrations of the proteins were calculated relative to the reference standard and expressed as relative abundance. Vertical scatter plot was plotted based on the values quantified and statistically compared using Mann-Whitney *U* test.

5.3.4.2 The Comparative Analysis of Candidate Protein Levels in DM and Other Neurological Disorders CSF

Five-µg of protein from CSF supernatants of IE, DM, MEN and cIVDD were utilised for 1-DGE and Western blot analyses. The experiment was executed as detailed in 4.3.3.3, page 117.

5.3.4.3 Age-related Influence on CSF Clusterin Levels

The upregulation of clusterin has been implicated in several neurodegenerative conditions mostly related to advanced aging (Sasaki et al. 2002; Calero et al. 2005). Therefore, the relationships of CSF clusterin levels with age were examined to exclude age-related changes as a potential variable. Correlation analysis was conducted on cases analysed in 5.3.4.2 using a non parametric, Spearman's rank correlation co-efficient (see 4.3.3.2, page 117).

5.4 Results

5.4.1 Identification of Potential Biomarkers in DM CSF through MALDI-TOF MS

The 1-DGE analysis of IE (*N*=4) and DM homozygotes CSF (*N*=4) is demonstrated in Figure 5-1. The comparison of protein profile between these two groups revealed a differentially expressed protein band estimated at 38kDa, which was consistently present in all DM samples and almost undetectable in IE CSF. The IE sample marked in lanes 5 and 7 displayed low protein sample which may be explained by protein loss during acetone precipitation or technical error during sample loading.

The upper and lower bands from a DM sample in lane 4 were excised and sent for MALDI-TOF analysis. Identification of the upper band revealed clusterin and apolipoprotein E proteins. In the lower band, clusterin was detected with haptoglobin protein. The information obtained from MASCOT peptide database is summarised in Table 5-1.

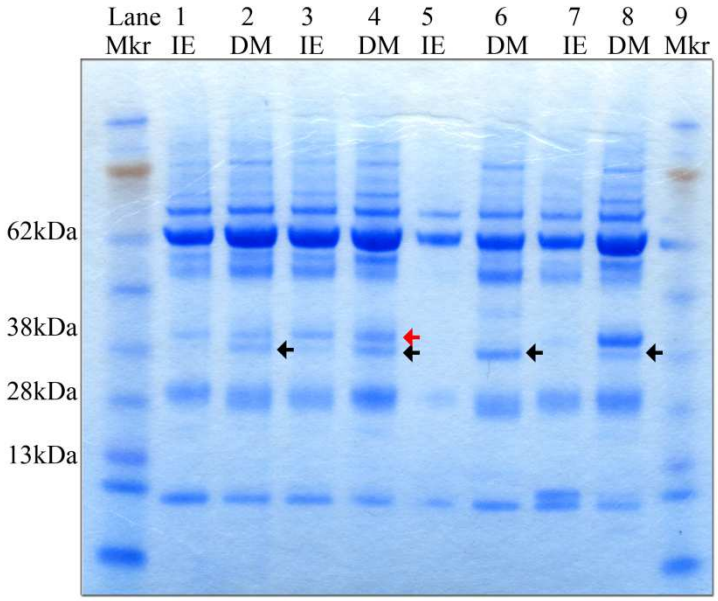

Figure 5-1: The 1-DGE analysis of IE and DM CSF.

One-DGE analysis of IE (*N*=4) and DM CSF (*N*=4) followed by Coomassie staining revealed an additional protein band at approximately 38kDa, which was consistently visible in DM CSF (as shown by black arrow), but present at a far lower intensity in the IE cases. Two bands, an upper (red arrow) and lower (black arrow) from a DM sample (lane 4), were excised and sent for MALDI-TOF analysis. Note the comparatively low densities of staining in lane 5 and 7 (IE).

Band	Mass	Peptide matched	Protein Score	Protein
Upper band	35332	2	123	Canine apolipoprotein E
	51757	2	115	Canine clusterin
Lower band	36434	3	151	Canine haptoglobin
	51757	2	90	Canine clusterin

Table 5-1: Proteins identified by MASCOT peptide database after in-gel trypsin digestion of protein bands from 1-DGE analysis. A protein score of more than 50 is considered a good identification.

5.4.2 Validation of Commercial Antibodies in Canine CSF

Antibodies against haptoglobin and clusterin were tested for their compatibility with canine CSF. Each antibody displayed a robust cross-reactivity with canine CSF (Figure 5-2). Western blot on haptoglobin revealed a single band estimated at 38kDa in canine CSF. Robust cross-reactivity was also observed in canine CNS tissue homogenates but not in murine brain tissue homogenates. Clusterin protein was found around 38kDa in Western blot, which was extremely robust in canine CSF but demonstrated only a weak signal in canine brain tissue homogenate even at 50µg of protein. Clusterin was not detected in mouse CNS tissue.

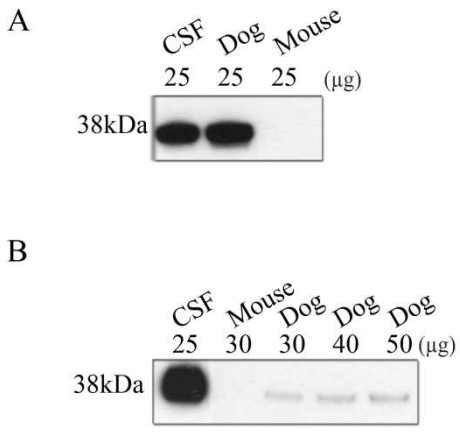

Figure 5-2: The validation of commercial antibodies of haptoglobin and clusterin in canine CSF using Western blot.

A) Haptoglobin: A robust signal at 38kDa is observed for canine CSF and brain tissue homogenate but not detected in mouse brain tissue. B) Clusterin: A robust signal at 38kDa is observed in canine CSF. A weak signal is detected in canine brain tissue but not in mouse brain tissue.

5.4.3 Pre-Analytical Assessment: Influence of Sample Handling Regimes on the Candidate Protein Levels in Canine CSF

The stability of haptoglobin and clusterin was assessed according to the protocols established for candidate proteins as described in 4.3.2, page 115. The protein profiles in 1-DGE was visualised by silver staining and revealed a consistent pattern between control and untreated samples (Figure 4-3). There was no sign of degradation observed. Aliquots from the samples generated during this study were stored and analysed for the novel protein described in this chapter. All samples marked "X" were excluded from statistical analyses due to the unquantifiable signals in Western blots. The complete list of dogs included in each analysis is given in Appendix 8.1.3.

5.4.3.1 Assessment of Haptoglobin Levels Following Treatment in Canine CSF

The effects of the three CSF sample handling practices on the levels of haptoglobin is summarised in Figure 5-3. The haptoglobin signals in lanes 7, 8, 15, and 16 were undetectable. This observation was consistent across treatment groups and therefore excluded from subsequent statistical analysis. One CSF sample in lane 5 in the 37°C group was also undetectable and excluded, which could be contributed by technical error while loading the samples. Statistical comparison of the levels of haptoglobin in various storage conditions did not differ statistically between treatment and corresponding control groups. Table 5-2 shows the means and standard deviations for each control and treatment group.

5.4.3.2 Assessment of Clusterin Levels Following Treatment in Canine CSF

The effects of the three CSF sample handling practices on the levels of clusterin is summarised in Figure 5-4. The clusterin signals in lanes 1, 2, 3, and 4 were consistently undetectable in all treatment groups and therefore excluded from statistical analysis. Clusterin intensity was unaffected by either 4°C or 37°C conditions but was significantly reduced ($P<0.05$) when the samples were incubated at room temperature for 48 hours. The numerical data for each control and treatment group are presented in Table 5-2.

Protein / Treatment	Haptoglobin	Clusterin
Control	8151±3223	12567±5156
4°C overnight	9234±3315	10400±3943
Control	7812±5295	16339±6450
37ºC for 4 hours	7490±5785	20881±9795
Control	8295±2233	19779±6391
RT for 48 hours	8866±3034	9258±7069*

Table 5-2: The optical density values for haptoglobin and clusterin in pre-analytical assessment.

Data presented as mean ± standard deviation and * corresponds to $P<0.05$.

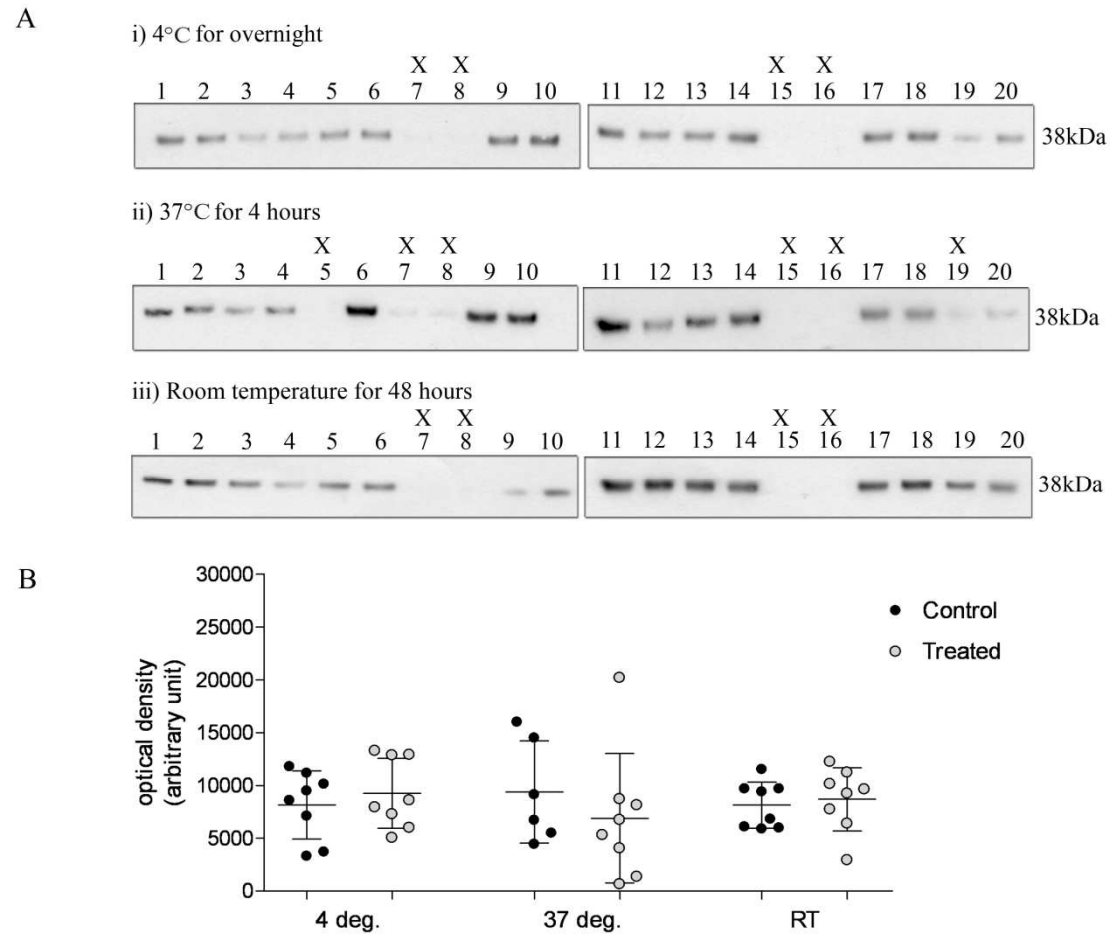

Figure 5-3: The influence of sample handling regimes on haptoglobin stability.

A) The haptoglobin Western blot signals for i) 4°C overnight ii) 37°C for 4 hours and iii) room temperature B) The optical densities were quantified, corrected for gel handling variations, expressed as arbitrary units, and plotted using vertical scatter plot. Haptoglobin levels are not altered under all conditions examined. Samples marked "X" were excluded from statistical analysis. Data presented as mean ± standard deviation.

Lane with odd numbers - control CSF; Lane with even numbers - treated CSF

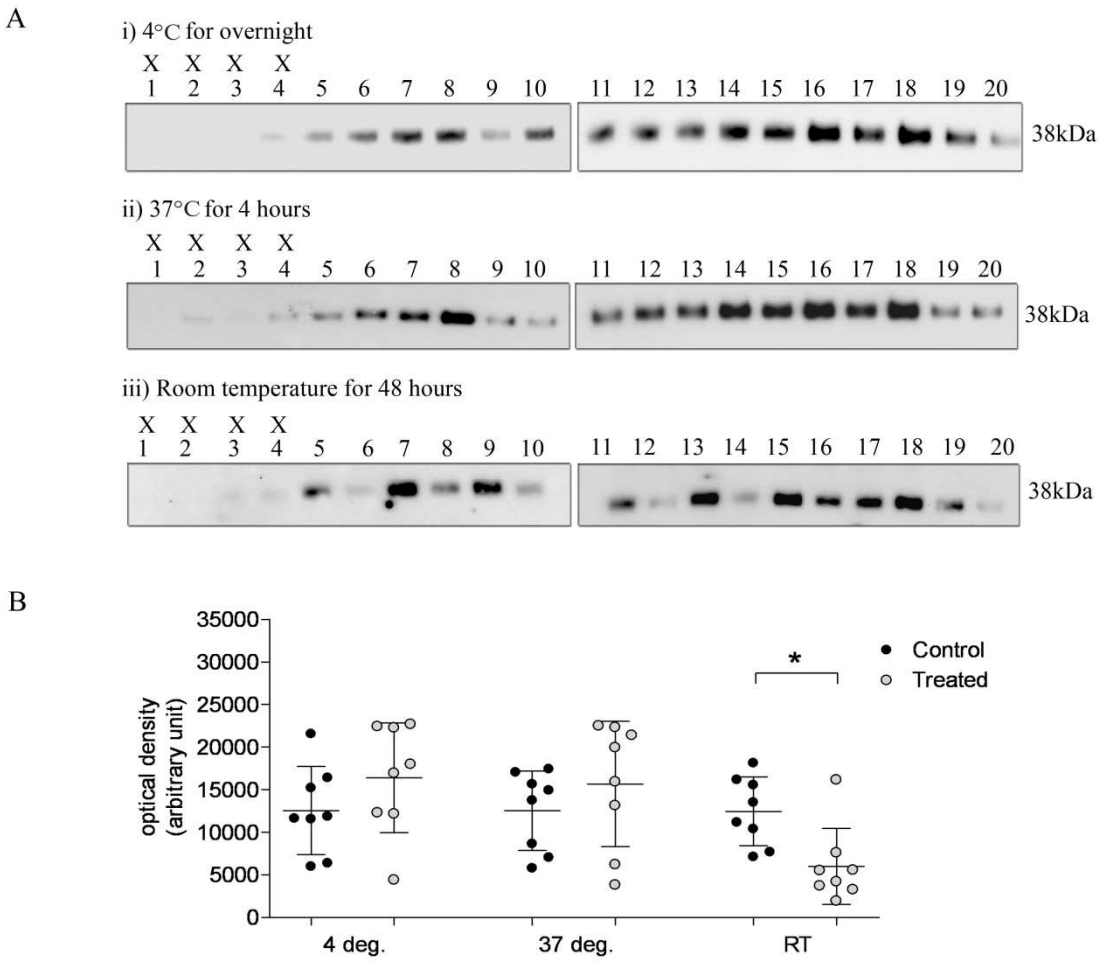

Figure 5-4: The influence of sample handling regimes on clusterin stability.

A) The clusterin Western blot signals (*N*=8) for i) 4°C overnight ii) 37°C for 4 hours and iii) room temperature. B) The protein optical densities were quantified, corrected for gel handling variations, expressed as arbitrary units and plotted using vertical scatter plot. Clusterin levels were not altered under 4°C and 37°C conditions, but appeared significantly reduced when treated at room temperature. Samples marked "X" were excluded from statistical analysis. Data presented as mean ± standard deviation, * represents $P<0.05$.

Lane with odd numbers - control CSF; Lane with even numbers - treated CSF

5.4.4 The Comparative Analyses of Candidate Proteins in DM and Other Neurological Disorders CSF

All CSF samples utilised for comparative Western blot analysis were checked for signs of protein degradation (data not shown).

Samples marked “X” were excluded based on the criteria outlined in 4.3.3, page 116. CSF sample collected from lumbar cistern was marked as “L” and the protein value was indicated by pink-coloured dot in the vertical scatter plot. Samples marked “Xs” were unable to be quantified, therefore were also excluded from further analysis.

5.4.4.1 The Comparative Analysis of Haptoglobin in IE and DM CSF

The quantification of haptoglobin levels in IE and DM revealed no significant differences between groups (Figure 5-5). CSF samples that had been treated with prednisolone at the point of CSF sample collection were excluded (marked with asterisk) as it is widely known that prednisolone administration can alter the haptoglobin levels (Willams et al. 1961; McConkey et al. 1979). The means and standard deviations for this experiment are given in Table 5-3.

5.4.4.2 The Comparative Analysis of Clusterin in IE and DM CSF

The relative concentrations of clusterin (Figure 5-6) revealed significantly increased clusterin in DM compared to IE group ($P<0.001$). The descriptive statistics for this analysis are presented in Table 5-3.

5.4.4.3 The Comparative Analysis of Clusterin in CSF from Selected Neurological Disorders

Results for the comparative study of clusterin in a range of neurological disorders are displayed in Figure 5-7. CSF clusterin was significantly elevated in DM compared to IE ($P<0.001$), which was consistent with the previous finding. In addition, CSF clusterin levels in DM were significantly elevated compared to MEN group ($P<0.05$). A similar pattern of CSF clusterin elevations was also observed in cIVDD cases ($P<0.01$) compared to IE cases. Although there was no significant difference detected in CSF clusterin levels between DM and cIVDD groups, the levels of clusterin was elevated by 20% in DM CSF compared to cIVDD cases. The numerical data is shown in Table 5-4.

5.4.4.4 Age-related Influence on the CSF Clusterin Levels

Spearman's rank correlation analysis revealed a weak, positive relationship between CSF clusterin levels and age in various neurological cases, however the relationships were not statistically significant (Figure 5-8).

Protein / Group	Haptoglobin	Clusterin
IE	32.8±23.5	59.9±38.7
DM	69.9±49.5	202.1±27.1***

Table 5-3: The haptoglobin and clusterin levels in IE and DM CSF

Data presented as mean ± standard deviations, values calculated in relative to reference standard. *** equivalents to $P<0.001$.

Protein / Group	Clusterin	*P* value
[a]IE	27.9±34.1	$P<0.001$** (a vs. b)
[b]DM	171.0±32.0	$P<0.05$* (a vs. c)
[c]MEN	93.2±46.5	$P<0.01$** (a vs. d)
[d]cIVDD	136.0±51.4	$P<0.05$* (b vs. c)

Table 5-4: The CSF clusterin levels in various neurological disorders.

Data presented as mean ± standard deviations, values calculated in relative to reference standard. * represents $P<0.05$, ** is $P<0.01$, *** is equivalent to $P<0.001$.

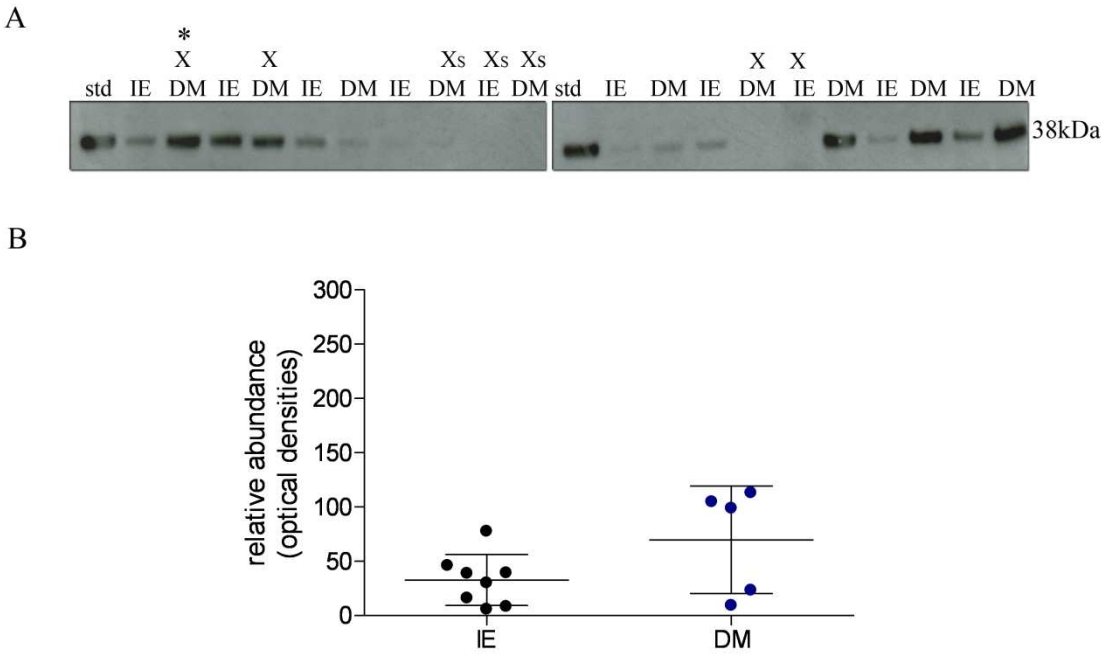

Figure 5-5: The comparative analysis of haptoglobin in IE and DM CSF.

A) The haptoglobin levels of IE (*N*=8) and DM (*N*=5*)* in Western blot analysis B) Vertical scattered graph was plotted to show the data distribution, revealed no significant difference in the means between groups. Case marked by asterisk (*) had been treated with prednisolone and was excluded from statistical analysis. Samples marked "X" and "Xs" were also excluded from statistical analysis. Data presented as mean ± standard deviation.

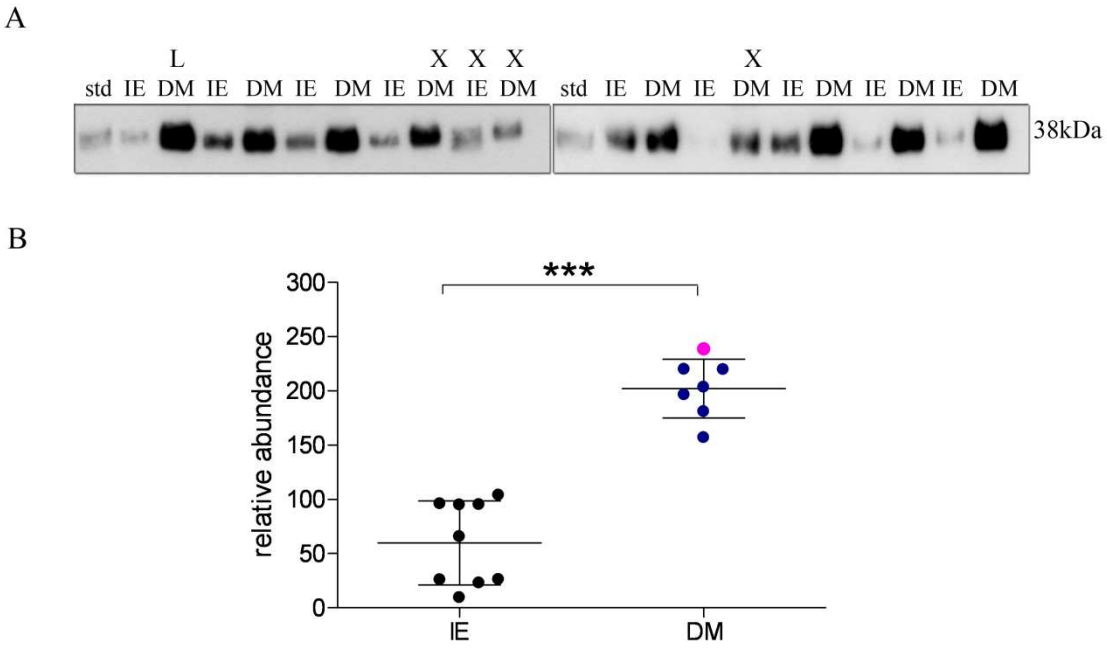

Figure 5-6: The comparative analysis of clusterin in IE and DM CSF

A) The clusterin levels of IE (*N*=9) and DM (*N*=7) in Western blot analysis B) Vertical scattered graph was plotted to show the data distribution and revealed significant elevation of clusterin in between IE and DM groups (*P*<0.001). Samples marked "X" were excluded from statistical analysis. Sample marked "L" was collected from lumbar CSF and the protein value from this sample is represented as pink-coloured dot in the vertical scatter graph. Data presented as mean ± standard deviation. *** represents *P*<0.001.

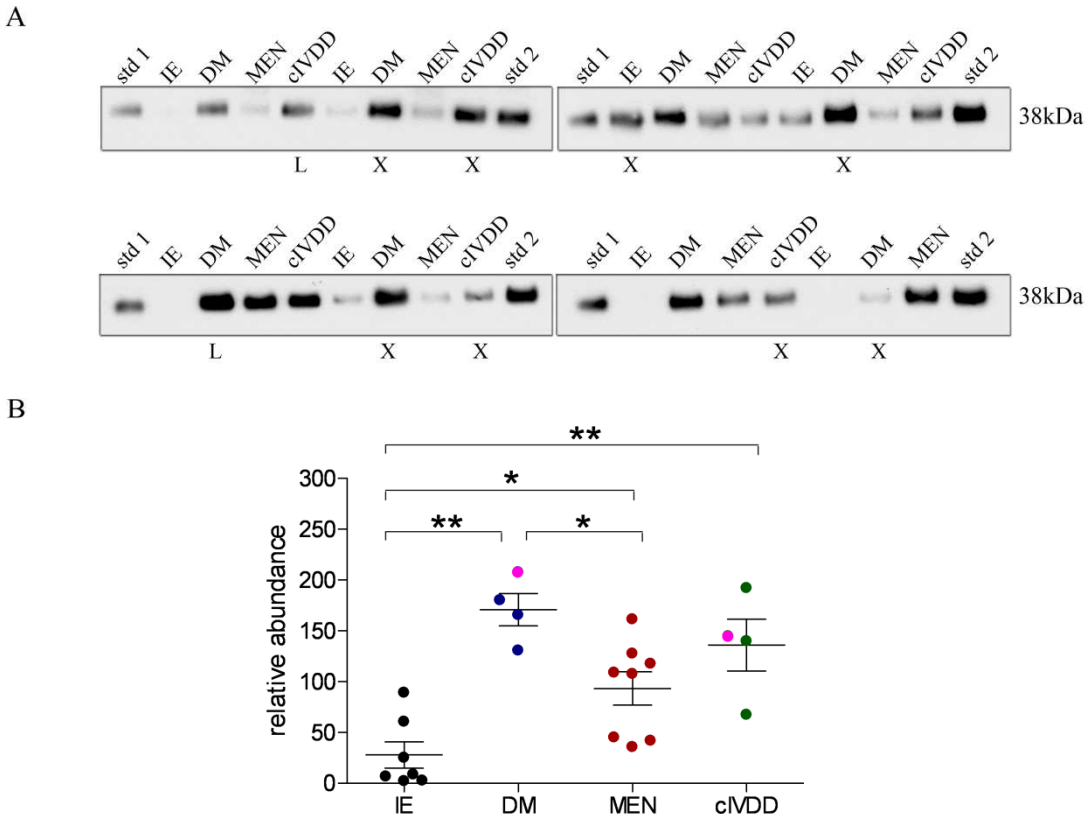

Figure 5-7: The comparative analysis of clusterin CSF in various neurological disorders.

A) Clusterin signals obtained from Western blot analyses. B) Signals were quantified and plotted in vertical scatter plot. Clusterin is elevated in DM (*N*=4) and cIVDD (*N*=4) compared to IE (*N*=7) (DM vs. IE, p<0.001; cIVDD vs. IE, p<0.01) and meningitis (*N*=8) (DM vs. meningitis, p<0.05; cIVDD vs. meningitis, p>0.05). There is no significant difference in CSF clusterin between DM and cIVDD. Samples marked "X" were excluded from statistical analysis. Sample marked "L" was collected from lumbar CSF and the protein value from this sample is represented as pink-coloured dots in the vertical scatter graph. Data presented as mean ± standard deviation. * represents *P*<0.05, ** is *P*<0.01, *** is equivalent to *P*<0.001.

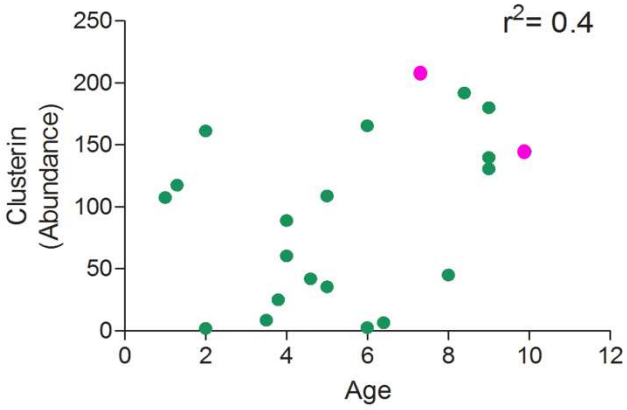

Figure 5-8: The data distribution of CSF clusterin levels in various neurological disorders.

The correlation analysis of CSF clusterin levels versus age demonstrate weak but positive relationship, however no significant relationship was detected. Protein values indicated by pink-coloured dots represent lumbar CSF.

5.5 Discussion

A combination of MALDI mass spectrometry coupled with TOF was employed in this study to identify proteins that are present within bands visualised on 1-DGE analysis. Haptoglobin and clusterin proteins were identified by MALDI-TOF MS analysis as major components of a visible band that appeared to be differentially expressed in DM CSF. The validation of haptoglobin and clusterin in canine CSF was performed by Western blot analyses using commercial antibodies. The cross-reactivity analyses of canine CSF was performed in tandem with tissue homogenates to assess species cross-reactivity and the migration characteristics in the different biological material.

5.5.1 Haptoglobin

Haptoglobin signals in canine CSF and dog brain homogenates were robust, (38kDa), however were not detectable in mouse brain tissue. The haptoglobin antibody was raised against canine haptoglobin, therefore it is most likely that the lack of cross-reactivity in mouse brain tissue is due to differences in sequence homology between canine and mouse haptoglobin. Biomarker specificity requires a clear demonstration that they are unaffected by circumstances related to sample collection and handling. An assessment of the haptoglobin protein stability was conducted using aliquots of IE CSF that had been generated in the previous study described in chapter 4. Haptoglobin appeared to be unaffected by any of the treatment regimes and to our knowledge the stability of this protein in CSF has not been previously reported at 4ºC, 37ºC or room temperature, although this protein is stable in saliva stored at -20 °C for 120 day (Gutierrez et al. 2011). Analysis of the effects of multiple freeze-thawing cycles on saliva haptoglobin has demonstrated an increment in haptoglobin concentrations (Gutierrez et al. 2011).

Haptoglobin is a major plasma glycoprotein synthesised in the liver and is present in most body fluids including CSF (Wang et al. 2001). In addition, haptoglobin is an acute phase protein, the levels of which are regulated by inflammatory processes, it has therefore generally been used as an indicator of infection, inflammation and trauma (Kushner and Rzewnicki, 1994). An elevation of CSF haptoglobin was observed in Huntington's disease, which could have been triggered as a compensatory mechanisms to pathogenic processes in Huntington's disease (Huang et al. 2011). The reduction of haptoglobin levels in human CSF has also been associated with Alzheimer's disease (Jung et al. 2008) although its

biological mechanism in this disease are unclear. To date no report has been found on CSF haptoglobin in ALS. The comparison of haptoglobin levels in IE and DM CSF by Western blot found that level differences between these groups were not statistically significant, even after the elimination of prednisolone therapy and exclusion of DM-affected heterozygotes as sources of variations. The lack of haptoglobin signals was also noted in few CSF samples, however this could be due to the low concentration of haptoglobin in CSF (reference ranges in human 0.06±0.009mg/dl compared to plasma 133.6±26.9mg/dl) (Chang et al. 2013). At this point, there is no indication that CSF haptoglobin is a biomarker of DM.

5.5.2 Clusterin

The characterisation of clusterin in canine CSF revealed robust protein signals, implying this protein is abundant in canine CSF, thus increasing the practicality of exploring clusterin as a potential biomarker for DM. Clusterin, also known as apolipoprotein J protein, is ubiquitously secreted in mammalian tissues and body fluids and is represented as α and β chains linked by disulphide bridges (Jones and Jomary, 2002). In our study, clusterin protein is detected in high levels in canine CSF at 38kDa, which has been confirmed as the β chain of clusterin through comparison of the protein sequences (Accession number: NP_001003370.1) with sequence information provided by the antibody's manufacturer. Clusterin has demonstrated weak signals in canine brain tissue homogenate, which may be suggestive of the low abundance of clusterin in canine brain, although it is later discovered in this project that clusterin is highly expressed in canine spinal cord tissue (see 6.4.3.3, page 177). In mouse brain tissue, clusterin is not detectable, which may be due to a lack of specificity of the commercial antibody.

In the pre-analytical study, clusterin in canine CSF was observed to be stable in 4°C and 37°C conditions. However, the prolonged exposure of CSF to room temperature for 48 hours resulted in a significant reduction in the levels of CSF clusterin. This reduction is potentially caused by the clusterin degradation or truncation into peptide fragments that could not be detected by the antibody however such degradation profiles can be characterised by high-throughput MS technologies (Carrette et al. 2005; Pieragostinoa et al. 2010). To our knowledge this is the first study to have assessed the stability of clusterin in CSF, although other types of apolipoprotein, (apolipoprotein E) have been reported to be stable in CSF at room temperature for 17 hours (Hesse et al. 2000). On the basis of these findings, it can be concluded that it in order to avoid misinterpretation of Western blot data

due to inappropriate sample handling, an appropriate protocol for sample collection and rapid storage should be adopted (further details in 7.1, page 190).

The preliminary Western blot of clusterin levels demonstrated a significant elevation of clusterin in DM CSF compared to the IE CSF. Clusterin is a heavily glycosylated protein with the capability to bind a range of molecules in different biological processes (Rosenberg and Silkensen, 1995; Jones and Jomary, 2002). Clusterin has been widely implicated in human neurodegenerative diseases including AD (Calero et al. 2005), PD (Sasaki et al. 2002) and ALS (Grewal et al. 1999). In Alzheimer's disease, evidence has highlighted the involvement of clusterin in the clearance and/or aggregation of Aβ in Alzheimer's pathway (Nuutinen et al. 2009). Clusterin has been found to be highly expressed in Alzheimer's brain tissue (Lidstrom et al. 1998) however the CSF clusterin levels in Azheimer's patients revealed conflicting results either increased (Sihlbom et al. 2008) or unchanged (Lidstrom et al. 2001). However the levels of the deglycosylated form of clusterin in Alzheimer's CSF has revealed a significant increment by 70% compared to native form, therefore implying that glycosylation may influence accurate quantification of clusterin. Clusterin has also been elevated in PD CSF (Prikrylova et al. 2010; Maarouf et al. 2012), particularly in patients with a disease duration less than two years (Prikrylova et al. 2010). There are no reports of CSF clusterin in ALS, although increased clusterin mRNA expression was detected in sporadic ALS patients in areas undergoing neurodegeneration (see detail 6.5, page 184) (Grewal et al. 1999).

The levels of clusterin in DM CSF were consistently elevated compared to other neurological disorders; IE and MEN cases. Clusterin is also moderately elevated in cIVDD CSF, suggesting that elevation of clusterin in CSF may not be unique to DM. However, CSF clusterin in DM is elevated by 20% compared to cIVDD CSF that may imply clusterin as a potential biomarker for DM, possibly associated with neuronal dysfunction and death. These findings also highlight the need for the identification of multiple biomarkers in DM to improve clinical diagnosis. In addition, since clusterin levels in canine CSF are elevated in aging associated disorders; DM and cIVDD, it is imperative to evaluate the age-related influence on clusterin in the cases analysed. The correlation analysis of CSF clusterin levels in various neurological disorders revealed no significant relationship with age, therefore increases its potential as a putative DM biomarker. There is also no clear association has been reported on CSF clusterin levels and age in human although one study has reported a significant increase of clusterin expression in human

pituitary gland due to aging (Ishikawa et al. 2006). Additional characterisation of clusterin and the proposed underlying mechanisms leading to the clusterin elevation and neurodegeneration in DM are further discussed in chapter 6 (see 6.5, page 183).

6 CSF Clusterin as a Potential Biomarker of DM

6.1 Background

Clusterin, also known as apolipoprotein J, was originally identified in ram rete testis, and named after its ability to form protein clusters within a variety of cell types (Blaschuk et al. 1983). Canine clusterin protein is encoded by full length mRNA (Acession number: NM_001003370.1), containing 445 amino acids (Accession number: NP_001003370.1). Two forms of clusterin have been described, a nuclear and a secreted form (Calero et al. 2005). The nuclear form of clusterin (50-53kDa) is normally expressed at very low levels and translated by shorter mRNA through alternative splicing mechanism (Calero et al. 2005). The precursor polypeptide for the secreted form is approximately 80kDa, which is cleaved at the N-terminal signal peptide that can be dissociated in two 40kDa chains under reducing conditions (Blaschuk et al. 1983). These subunits are linked together by five disulfide bridges with six glycosylation sites (Calero et al. 2005). Clusterin is widely expressed in mammalian tissue including nervous tissue such as the choroid plexus (Aronow et al. 1993), brain (Oda et al. 1994), spinal cord (Danik et al. 1993). At the cellular level, clusterin has been shown to be present in astrocytes and neurons (Pasinetti et al. 1994; Xu et al. 2000). It is also abundantly secreted in nearly all body fluids, including plasma (De Silva et al. 1990), serum (Kapron et al. 1997), urine (Aronow et al. 1993) and CSF (Nilselid et al. 2006). Clusterin has been implicated in a number of diverse biological processes, including cell-cell interactions (Fritz et al. 1983; Silkensen et al. 1995), cellular stress responses (Rosenberg and Silkensen, 1995; Michel et al. 1997), sperm maturation (Sylvester et al. 1991), apoptosis (Buttyan et al. 1989; Viard et al. 1999) complement inhibition (Murphy et al. 1988), lipid transport (De Silva et al. 1990), tissue remodelling and membrane recycling (Danik et al. 1991), cellular debris clearance (Bartl et al. 2001) and extracellular matrix degradation through membrane-type 6 matrix metalloproteinase (Matsuda et al. 2003).

Clusterin has been implicated in various diseases ranging from reproductive cancers, cardiovascular and neurodegenerative diseases (McLaughlin et al. 2000; Moretti et al. 2007; Nuutinen et al. 2009). In veterinary medicine, clusterin has been characterised in the reproductive system and urinary tract (Ibrahim et al. 1999; Garcia-Martinez et al. 2012). A recent proteomic study has also identified serum clusterin in canine lymphoma (Atherton et al. 2013). Clusterin in this study is found to be robust in canine CSF and markedly elevated in DM CSF (see 5.4.4.3, page 153). This chapter therefore further describes the

expression of clusterin in DM through the examination of spinal cord tissue and plasma that may be the possible origin of clusterin in CSF.

6.2 Aims

The aim of the work presented in this chapter was to further evaluate clusterin as a potential CSF biomarker in DM and to establish whether its origin can explain the clusterin elevation in DM CSF. The specific aims were to:

1. Perform comparative evaluations of the plasma clusterin levels in IE and DM by Western blot

2. Compare clusterin mRNA expression in spinal cord in control and DM homozygotes by RT-PCR.

3. Characterise and compare clusterin distribution in control and DM spinal cord tissues by IHC.

6.3 Materials and Methods

6.3.1 The Comparative Analysis of Clusterin in IE and DM Plasma

6.3.1.1 Preparation of Plasma Samples

The extraction of plasma was performed using EDTA blood that was kept in -80°C. The blood samples were thawed on ice, and subsequently centrifuged at 1500rpm for 10 minutes. Plasma supernatants were transferred into fresh 1.5ml eppendorf tube and were diluted at 1:20 with ultrapure water. The total protein concentrations for these plasma samples were not determined due to the high content of haemoglobin. Three-µl of diluted plasma was prepared and denatured (see 2.3.4, page 80) for 1-DGE as described in 2.3.5, page 80. Five-µg of protein from DM CSF sample was also included for comparison.

6.3.1.2 Polyacrylamide Gel Preparation (Large Format)

Two large format, hand-poured gels (16cm X 13cm) were used in this study. Resolving gel at 12.5% was prepared from a stock of acrylamide:bisacrylamide (ratio 37:1, Sigma, UK)

mixed in resolving gel master-mix containing 1.5M Tris pH8.8, 10%SDS, 10% ammonium persulphate, TEMED, (Appendix 8.2.6). Forty-ml of the gel solution was added into the gel cassettes, followed by the addition of 500µl of 0.1% SDS solution on the top of resolving gel to prevent oxidation at the surface of the polyacrylamide gel. The resolving gel was allowed to polymerise for two hours. The 0.1% SDS was discarded and subsequently, 4% (10ml) of stacking gel solution (Appendix 8.2.6) was added to the polymerised gel. A 20-well comb was inserted and the stacker was allowed to polymerise for one hour. Once the stacker was polymerised, the comb was removed and the wells were rinsed with 0.01% SDS solution. The gel cassettes were placed in the gel rig and subsequently filled with 1X running buffer containing 25mM Tris, 192mM glycine, 0.1% SDS, pH8.3 (Appendix 8.2.8). The protein samples were loaded into the wells and electrophoresed for five hours at 250mA.

The gel was removed from the gel cassettes. The first gel was washed with ultrapure water for 10 minutes and stained with silver stain as detailed previously in 2.3.6, page 81. The second gel was processed for Western blot.

6.3.1.3 Electrophoretic Transfer and Western Blot Analysis

The electrophoretic semi-dry transfer was performed based on Towbin method (Towbin et al. 1979). Preparation of cathode and anode buffers for semi-dry electrophoretic transfer is described in Appendix 8.2.7. The gel was briefly rinsed with ultrapure water for five minutes and then equilibrated in Towbin cathode buffer under gentle agitation for five to 10 minutes. A transfer stack was prepared; 1) filter papers that were pre-soaked in anode buffer (bottom electrode), 2) nitrocellulose membrane (Millipore, UK), pre-soaked in anode 2 buffer, 3) polyacrylamide pre-soaked in cathode buffer, and 4) filter papers that were pre-soaked in cathode (top electrode). The protein was transferred to nitrocellulose membrane using a semi-dry blotter system (Bio-Rad, UK) for two hours at 250mA.

The membrane was stained with Ponceau S (see 2.3.7, page 81) to check for protein loading and subsequently washed with 1X T-TBS buffer (Appendix 8.2.5). The membrane was blocked overnight at 4°C with 5% milk powder in 1X T-TBS buffer. The membrane was washed three times with 1X T-TBS (5 minutes/wash) and incubated with anti-clusterin antibody at 1:100,000 dilution (see Table 2-1) in 5% milk powder in 1X T-TBS. Following incubation with secondary antibody, the membrane was exposed to Hyperfilm

and immuno-complex signal detected by ECL reagent were quantified as described in 2.3.7.1, page 82.

Vertical scatter graph was plotted based on these arbitrary values. Statistical comparison of the data between IE and DM group was conducted using Mann Whitney *U* statistical analysis (see 2.6, page 87).

6.3.2 The Comparative Assessment of Clusterin mRNA Levels in Control and DM Cases

6.3.2.1 CNS Material for RNA Extraction

The RNA extraction was conducted in control and DM homozygotes using T12 spinal cord as detailed in 2.4.2, page 84. The diagnoses of each DM and control case had been confirmed in a previous study (Johnston, 1998). The selection of DM material was achieved by routine clinical diagnostics, histopathological examination and the homozygosity of the mutant allele in *Sod*1 gene (see 3.4.1.1, page 94). Controls were sourced from non-neurological cases with a wild type *Sod*1 gene profile. The complete list of dogs included in this analysis is given in Appendix 8.1.2. Blood and CSF samples were not available for these cases.

Following RNA extraction, the RNA yield was quantified (see 2.4.3, page 85) and the quality of RNA was assessed by 2% of ethidium bromide stained agarose gel (see 2.4.4, page 85).

6.3.2.2 cDNA Generation from total RNA

Since RNA is fragile and more easily degraded than DNA, the measurement of mRNA levels was achieved through synthesis of cDNA using a method known as reverse transcription (RT reaction) and amplification of cDNA by RT-PCR. cDNA synthesis was performed with the presence of reverse transcriptase enzyme, where the RNA is reverse transcribed into cDNA that are complementary to mRNA transcript, which later can be used as template for RT-PCR.

Single strand cDNA was synthesised from total RNA by reverse transcriptase reaction using reagents supplied by Invitrogen (UK). Two-µg of total RNA was incubated at 65°C

for five minutes and the RNA quickly chilled on ice. This step is to denature any secondary structures that might disrupt the transcription as well as linearising the RNA. The reverse-transcriptase (RT) was carried out by adding a mixture of 5X RT buffer at pH 8.3 (250mM Tris-HCI, 375mM potassium chloride, 15mM magnesium chloride), 0.1mM DTT, 20mM each of dNTP (dATP, dGTP, dTTP, dCTP), random primers, 20 units RNAse inhibitor, and 200 units RT enzyme to the sample tube. The reactions were incubated at a sequence of temperatures which have been established in our group to yield efficient cDNA production; 37°C for 30 minutes, 42°C for 1 hour, and 72°C for 15 minutes. The re-suspension of cDNA was carried out by adding ultrapure water to give a final volume of 100µl.

6.3.2.3 Primer Design

The forward (5'-GCC CTT CTT TGA CAT GAT ACA CCA-3') and reverse (5'-TGC TTC TGG GAT CAT CAC CGT GA-3') primers (Eurofins, Germany) for PCR were designed using an interactive web-based primer program, GeneFisher software version 1.2.2 (BiBiServe, Germany). These primers were used to amplify sequences based on the canine clusterin mRNA sequence (NM_001003370.1) which was obtained from the online public database. The amplification of cDNA by RT-PCR would specifically generate a product with 500 base pairs nucleotide.

6.3.2.4 Standardisation of cDNA Utilising Housekeeping Genes

A housekeeping gene, cyclophilin was utilised as an internal standard. The forward (5'-ACC CCA CCG TGT TCT TCG AC-3') and reverse (5'- CAT-TTG-CCA-TGG-ACA-AGA-TG-3') primers were obtained from a previous study (Danielson et al. 1988). The cyclophilin message was used as a reference standard control as the levels of message in tissues are expressed constantly and are not altered under experimental conditions. The PCR was set up using a pre-setting RT-PCR programme; 34 cycles, 94°C for 2 minutes, 94°C for 30 seconds minute, 58°C for 30 seconds, 72°C for 2 minutes, 72°C for 5 minutes. Four-µl of RT-PCR products were visualised using 2.5% ethidium bromide stained agarose gel and were examined under ultra violet light (GeneFlash, Syngene, USA). The signals were quantified using Scion Image NIH.

6.3.2.5 RT-PCR

Four-µl of cDNA was utilised for RT-PCR using the same programme as with cyclophilin (see 6.3.2.4, above) with the addition of 2.5µl DNA RedTaq® ReadyMix™ buffer (Sigma-Aldrich, UK), 0.5µl of each primer (10pmol/ul) and ultrapure water. The visualisation and quantification of RT-PCR products was achieved as described in 6.3.2.4. The data normalisation was performed through the comparison of the cyclophilin signals with RT-PCR products generated from clusterin cDNA. The normalised values, which reflect the clusterin mRNA levels, were analysed using Mann Whitney *U* test to determine if there was any difference in mRNA expression between control and DM group.

6.3.3 IHC Analysis of Clusterin in Controls and DM Spinal Cords

6.3.3.1 Archival Paraffin-embedded Blocks

Archival paraffin-embedded blocks were utilised for IHC analyses. Spinal cord tissues for paraffin blocks were sourced from the same cases in 6.3.2.1, page 167 processed and embedded in paraffin wax for a previous PhD study (Johnston, 1998). However, since these paraffin embedded tissue blocks were prepared for a microtome that is no longer available, all blocks had to be re-processed and re-embedded with paraffin wax. Paraffin blocks were melted down and run through the wax cleaning cycle on a preset programme of 27 minutes on an automated tissue processor machine (Thermo Fisher Scientific, UK). The blocks were then re-embedded on the Tissue-Tek® VIP® (Sakura, USA) (Appendix 8.4.3). The re-processed paraffin blocks were cut at 4µm thickness with a microtome (Shandon Finesse®, Thermo Scientific, UK) and mounted onto the silane-coated slides (see 2.5.1, page 86). The slides were dried at 60ºC for an hour and were baked at 37°C overnight.

Since the spinal cord material for immunohistochemistry was sourced from an archive of paraffin blocks, fresh tissue specimens of spinal cord from T12 spinal cord was also included and utilised as quality control (see 2.5.2.1, page 86). The spinal cord tissues were derived from a five year old, female, miniature Schnauzer that was euthanised due to acute paraplegia. The histopathological diagnosis of this case was hemorrhagic myelomalacia. The fresh spinal cord tissues were fixed and processed for paraffin wax embedding as described in 2.5.2.1, page 86. This tissue material will be referred to as “reference standard”.

6.3.3.2 Assessment of Tissue Morphology by H&E Staining

Following paraffin re-embedding of the archival tissues, all archival sections from T12 spinal cord were routinely stained with H&E to assess the overall tissue morphology (see 2.5.3, page 87). A reference standard was also included.

6.3.3.3 Optimisation of Clusterin Antibody for IHC

The optimisation of clusterin antibody (Abcam, UK) for IHC was performed using paraffin-embedded sections prepared from the reference standard.

The optimisation of clusterin IHC was conducted using a commercial kit, Envision+™ System HRP (Dako Cytomation, UK) in a range of dilutions; 1:500, 1:1000, 1:2000, 1:4000, 1:8000, 1:16,000, 1:32,000 and 1:64,000. This system is extremely sensitive and based on the conventional peroxidase anti peroxidase system. Negative controls were prepared as appropriate with Dako universal diluent, *in lieu* of primary antibodies. Sections were initially dewaxed in histoclear, hydrated with 70% absolute alcohol, 70% methylated spirit and subsequently rinsed in water (Appendix 8.4.2). Antigen unmasking was performed using 10mM sodium citrate buffer pH6.0 (Appendix 8.4.5), in automated pressure cooked (Menarini Access Retrieval unit, Menarini Diagnostics, UK) for 1 minute 40 seconds at 125°C. The endogenous peroxidase activity was quenched by covering sections with 150µl peroxidase blocking solution (Dako Cytomation, UK) for five minutes and then washed with 1X TBS, pH7.5 (Appendix 8.4.6). Sections were then incubated with the primary antibody diluted in universal diluent containing 50mM Tris-HCl buffer with 1% BSA for one hour. The slides were washed twice with TBS buffer followed by addition of secondary antibody (rabbit anti goat) diluted in universal diluent buffer. After two washes, HRP conjugated antibody was added to the slides and incubated for 30 minutes. The sections were incubated twice for five minutes with 3,3'-Diaminobenzidine (DAB) chromogen, which produces brown coloured deposit in positive staining. Sections were washed in running water and counterstained in Gills haematoxylin. Following counterstaining, sections were blued in Scots tap water, dehydrated with 70% methylated spirit and 70% alcohol, cleared in Histoclear and mounted in distyrene plasticizer xylene (DPX).

6.3.3.4 Comparative IHC Analysis of Clusterin in Control and DM

The IHC comparison of clusterin was performed in archival sections from controls and DM homozygotes. Details on controls and DM cases included in the IHC study are given in (Appendix 8.1.2). Blocks were cut at 4µm, and stained with clusterin antibody at the pre-determined dilution as described previously (see 6.3.3.3, page 170). A reference standard section was also included in this experiment. Negative control was included by omitting the primary antibody. All sections were blindly assessed by the author and an independent reviewer who has experience in histological studies. Since quantitative analysis is not practical due to time limitation, a scoring system was devised to allow a more objective assessment between controls and DM (see Table 6-1). Vertical graphs were plotted and data was analysed using Mann Whitney *U* test.

Score	Grade	Intensity of clusterin staining in neurons
0	None	No positive staining
1	Very light	Very light positive staining in most cell bodies, which difficult to differentiate from the background
2	Light	Light brown staining in most cell bodies, but easily distinguish from the background. Punctate pattern is difficult to differentiate.
3	Moderate	Moderate positive staining. Punctate pattern is clearly visible in some cell bodies
4	High	Dark positive staining with punctate pattern scattered in the many cell bodies
5	Very high	Very intense positive staining. Punctate pattern is clearly visible in all cell bodies

Table 6-1: Parameter used for scoring clusterin IHC analysis.

6.3.3.5 Neuron-specific Enolase Staining

Neuron specific enolase (NSE) is a glycolytic enzyme that presents in central and peripheral neurons as well as neuroendocrine cells, therefore it serves as a neuronal marker in IHC. NSE staining was conducted on each control and DM section to evaluate the density and distribution of the neurons and neuronal cell bodies. Positive staining is identified as brown with more intense staining usually localised in neuronal cell bodies. All

sections were stained with mouse monoclonal anti NSE (Dako Cytomation, UK) at 1:1000 dilution and subsequently with HRP rabbit anti mouse secondary at 1:100 dilution using the protocol described in (see 6.3.3.3, page 170).

6.4 Results

6.4.1 The Comparative Analysis of Clusterin in IE and DM Plasma

All DM-affected dogs with a heterozygous genotype were excluded from statistical analysis (marked as "X"). The assessment of the protein profile using silver staining displayed comparable protein content, suggesting good sample loading. There was no evidence of gross protein degradation (Figure 6-1A). Western blot analysis of IE and DM plasma had shown that the molecular weight size of clusterin in plasma was comparable with CSF clusterin at 38kDa (lane 1). A proportion of DM cases (3/5) demonstrated a gel shift which may indicate a post translational modification (see Figure 6-1B) however this gel shift was also observed in an IE and a heterozygous case (marked by black arrow). Statistical analysis comparing IE and DM homozygotes demonstrated no significant difference in the protein level (M±SD for IE=98790±5561; M±SD for DM=10441±5139).

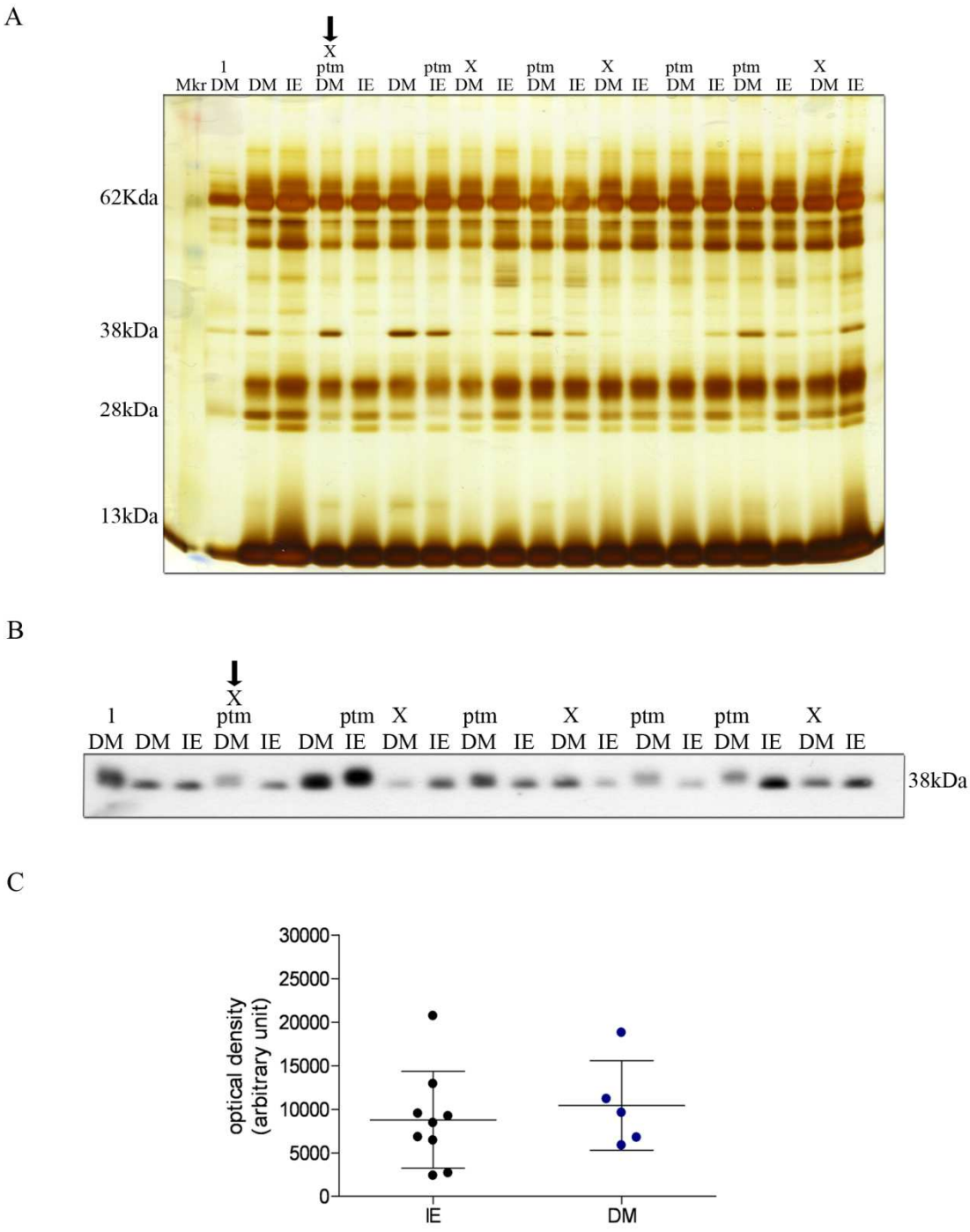

Figure 6-1: The assessment of plasma clusterin levels in IE and DM.

A) The global protein content across all samples in the silver stained gel is comparable with no obvious signs of gross protein degradation in any of the plasma samples. B) The band observed at 38kDa in the plasma samples has a similar migration distance to clusterin which has also a similar molecular weight with CSF clusterin (lane 1). Three out of five DM homozygotes display a gel shift of clusterin indicating post-translational modification (ptm), although a sample from IE and a DM heterozygous (as shown by black arrow) also exhibit a gel shift. C) Plasma clusterin signals were plotted in vertical scatter plot, expressed in arbitrary units. Statistical analysis between IE (*N*=9) and DM (*N*=5) reveal no significant difference. All DM heterozygote cases (marked as "X") were excluded from statistical analysis. Data presented as mean ± standard deviation.

6.4.2 The Comparative Assessment of Clusterin mRNA Levels in Control and DM Cases

The expression of clusterin mRNA in control and DM spinal cords was determined by RT-PCR using cDNA as a template. The RT-PCR signals were quantified and expressed in arbitrary units. The signals for cyclophilin and RT-PCR products were robust (Figure 6-2A). The RT-PCR signals from clusterin were normalised by expressing them as a density relative to cyclophilin, which serves as a reference standard based on its role as a house-keeping gene. Vertical scatter graph was plotted and demonstrated that the mean clusterin mRNA level was elevated by 42% in DM (M±SD=1.87±0.33) compared to control cases (M±SD=1.123±0.30), a difference bordering on statistical significance (P=0.05).

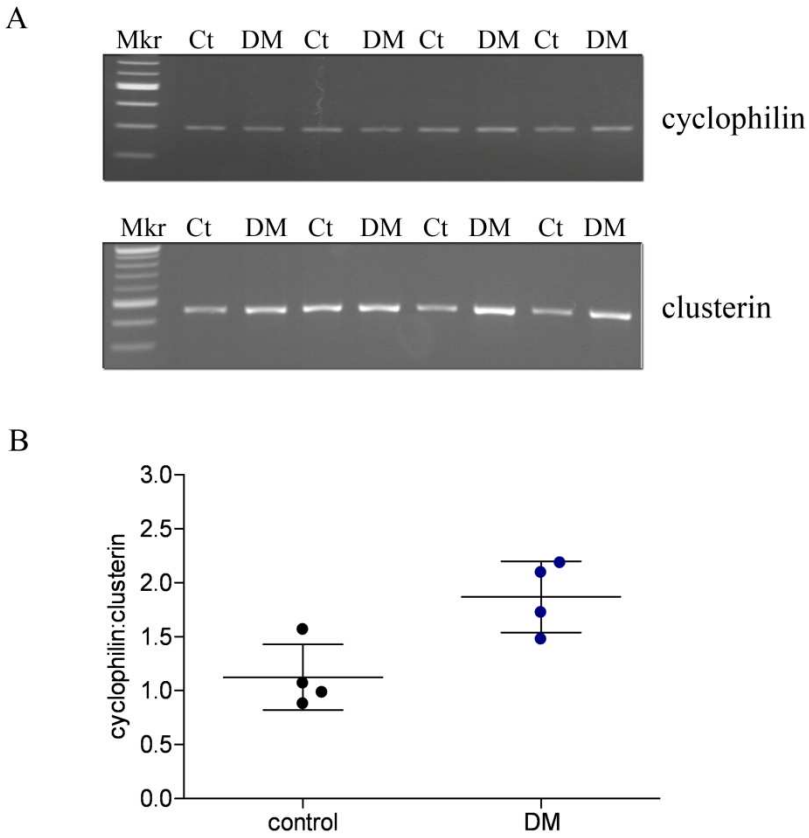

Figure 6-2: The comparison of clusterin mRNA levels in control and DM spinal cords.

A) The RT-PCR amplification of cyclophilin and clusterin cDNA in ethidium bromide stained agarose gels (2%) demonstrate robust signals for quantification. B) The signals for clusterin mRNA were normalised relative to cyclophilin signals (cyclophilin:clusterin) and plotted in vertical scatter graph. The statistical analysis revealed no significant difference between two groups (exact *P* value=0.05), however the mean of clusterin mRNA in DM group (*N*=4) was found to be elevated by 42% compared to control group (*N*=4). Data presented as mean ± standard deviation.

6.4.3 IHC Analysis of Clusterin in Controls and DM Spinal Cords

6.4.3.1 Assessment of Tissue Morphology by H&E Staining

The diagnoses for each case were determined in a previous study that was conducted in 1998. Therefore, the histopathological examinations were repeated on the re-processed sections stained with H&E. All sections were blindly assessed and the diagnosis of each case was confirmed by a veterinary pathologist, Dr. Pamela Johnston. In addition, the H&E staining was conducted to ensure that tissue morphology in archival material was not affected by the paraffin re-embedding with particular attention given to the morphology of neuronal cell bodies in the gray matter (Figure 6-3). The H&E staining in the archival sections were compared with a reference standard (Figure 6-3A and B). The paraffin re-embedding had minimal effect on the shape of the spinal cord, although slight distortion in spinal cord and gray matter was observed in a section from archival control (Figure 6-3C). At a higher magnification (10X) of the ventral horn area, the neuronal cell bodies in archival sections were large and intact.

6.4.3.2 Optimisation of Clusterin Antibody for IHC

Optimisation of IHC was performed using serial dilutions of the clusterin antibody and is demonstrated in Figure 6-4. This analysis was performed using sections obtained from the reference standard. Positive immuno-reaction was visualised with chromogen substrate DAB which produces brown staining. The highest concentration of 1:250 gave extremely high background staining in white and gray matter although it appeared that the dark brown staining was localised to neuronal cell bodies. Background staining in the gray matter was reduced at 1:4000 but still intense. Optimal staining was observed at 1:8000 dilution, where the positive brown staining was clearly localised in the neuronal cell bodies with minimal background staining in white and gray matter. This dilution was selected as the optimal dilution for this antibody. The positive brown staining had become faint at 1:32,000 and completely disappeared at 1:64,000 dilution.

When these conditions were applied to an archival section very light positive staining localised in the neuronal cell bodies could be seen (Figure 6-5B). Therefore 1:8000 antibody dilution was determined suboptimum for these archival materials, which could reflect the duration of tissue fixation and paraffin re-processing (Figure 6-5). A higher concentration was therefore selected for the archival sections. At 1:4000 dilution, minimal

background staining was identified and the positive staining was specifically detected in the neuronal cell bodies, recognised as dark and punctate appearance within the neuronal cell bodies. Therefore, 1:4000 dilution was used for archival spinal cord sections.

6.4.3.3 Comparative IHC analysis of Clusterin in Archival Control and DM Sections

Comparative IHC analysis of clusterin was carried out in the re-processed archival sections to evaluate the clusterin distribution in control and DM homozygotes (Figure 6-6A and C). Clusterin IHC demonstrated strong immuno-reactivity in both control and DM cases, consistently recognised as dark stain with a punctate pattern within the neuronal cytoplasm that may reflect aggregates containing the clusterin epitope (Figure 6-6E). Subjective assessment using a scoring system (Table 6-1) of the staining pattern consistently found that the positive staining was strictly confined within neuronal cell bodies, however there was no significant difference detected in staining intensity between control and DM groups (Figure 6-7).

NSE staining in archival and recently processed tissue demonstrates that the positive staining was identified throughout the white and gray matter although more intense staining was found localised in the neuronal cell bodies (Figure 6-6B and D). This confirms the localisation of clusterin in the neuronal cell bodies. However, since the sections for NSE staining were not obtained adjacent to sections for clusterin IHC, the distribution of the neurons were not identical.

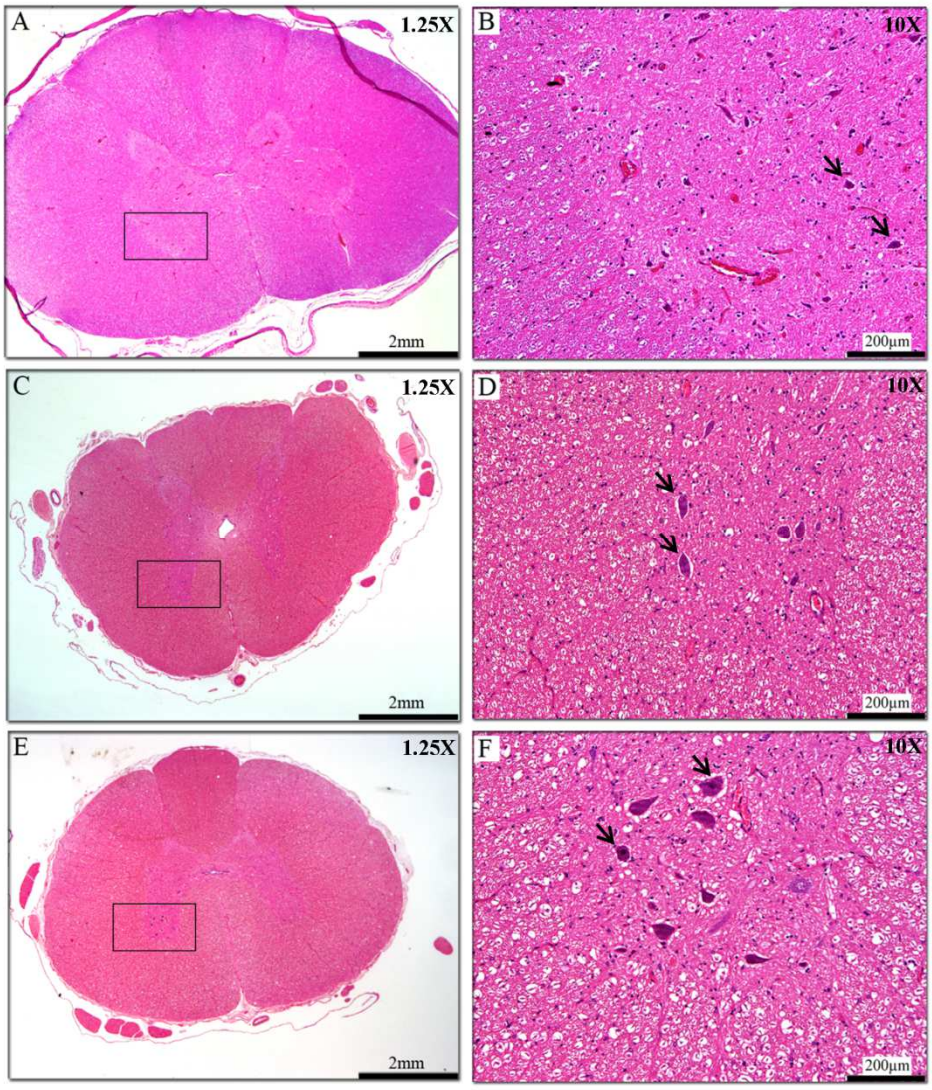

Figure 6-3: The cross-sections of T12 spinal cord stained with H&E.

A) Reference standard at 1.25X magnification. B) Reference standard at 10X magnification. C) Archival control section at 1.25X magnification. D) Archival control section at 10X magnification. E) Archival DM section at 1.25X magnification. F) Archival DM section at 10X magnification.

At 1.25X magnification, the overall shape of the spinal cord in C) and E) archival control and DM sections are minimally affected by the re-processing, although slight distortion is observed on the spinal cord shape and the gray matter in C) compared to the reference standard in A). 10X magnification of the ventral horn area in archival sections; D) and F) demonstrate the large and intact neuronal cell bodies (marked by arrow). The ventral horn area is indicated by box in section A), C) and E).

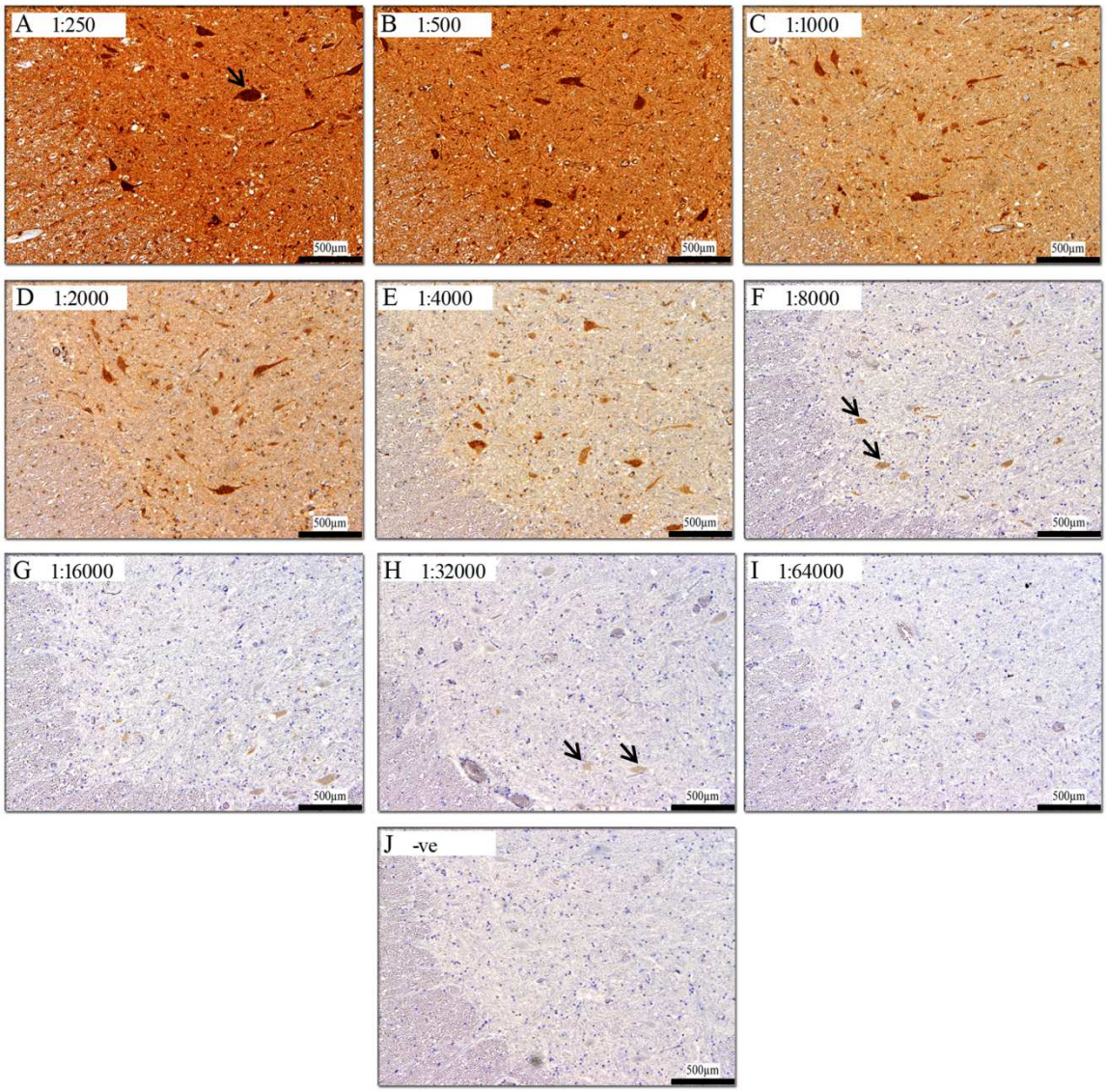

Figure 6-4: The dilution optimisation of clusterin antibody for IHC.

A) - I) IHC analysis on T12 spinal cord sections prepared from fresh fixed tissue/reference standard demonstrates the staining pattern over serial dilutions of the clusterin antibody, from 1:250 to 1:64,000 dilution. Extremely high background staining is observed throughout the white and gray matters at 1:250 (A) dilution. Even at highest concentration, the dark brown staining is seems to localise in the neuronal cell bodies (as shown by arrow). Moderate background staining remains at 1:4000 dilution. The positive brown staining in neuronal cell bodies can be clearly differentiated in this section (E). The optimum dilution is determined to be 1:8000 dilution, with a significantly reduced background. Note that the positive staining still can be observed at 1:16000 and 1:32000 dilution. J) represents the negative staining obtained in the absence of the primary antibody on a section which was processed at the same time. All the images were captured from ventral horn area at 10X magnification.

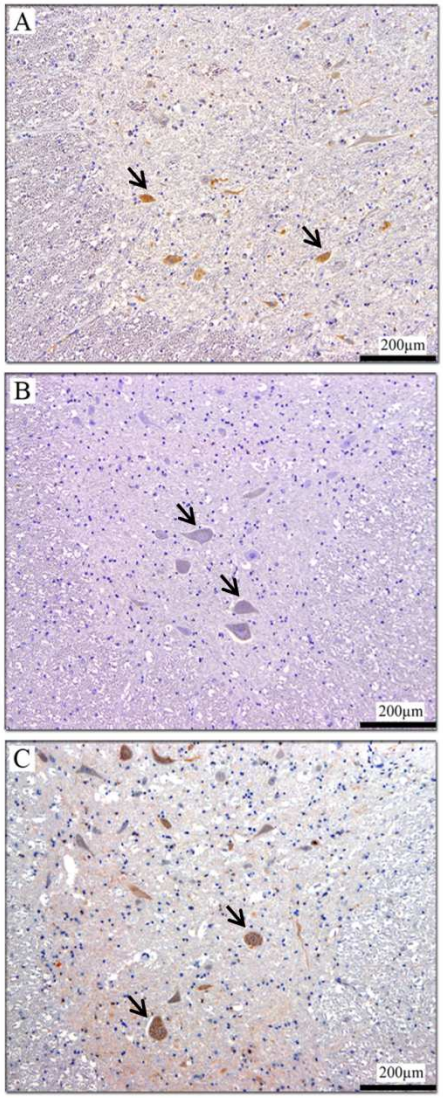

Figure 6-5: IHC analysis of clusterin at 1:8000 dilution is suboptimal for re-processed archival sections.

A) The optimum dilution in T12 spinal cord section prepared from reference standard was determined at 1:8000 dilution. Positive brown staining is found to be localised within neuronal cytoplasm. Minimal background staining is detected throughout the white and gray matters. B) Dilution at 1:8000 demonstrated very light positive staining within neuronal cell bodies in one of the archival control section, which barely differentiated from the background. C) Dilution at 1:4000 was determined as optimal for re-processed archival sections with minimal background staining detected. The positive staining is clearly seen in neuronal cell bodies, visualised as dark and punctate appearance within the neuronal cell bodies. Images were captured from ventral horn area at 10X magnification. Neuronal cell bodies were indicated by arrow.

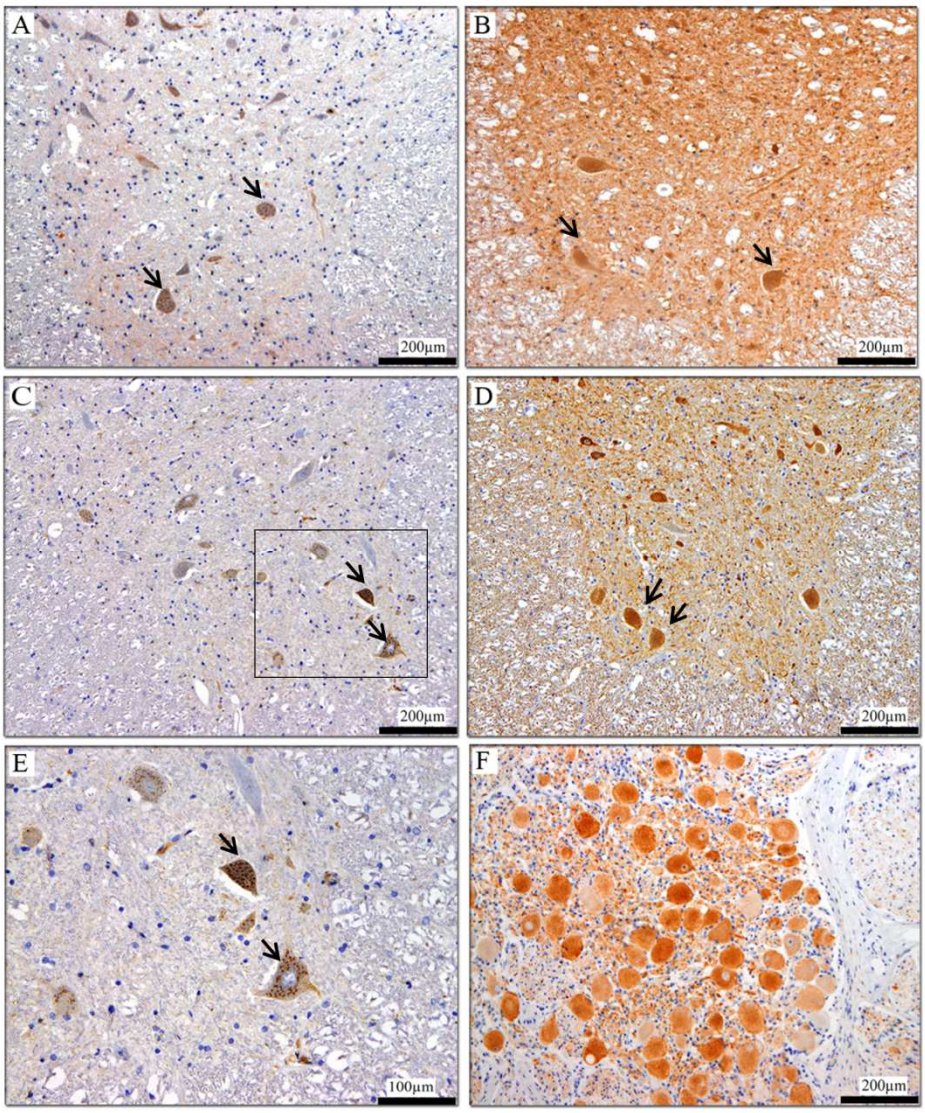

Figure 6-6: The clusterin and NSE staining in archival control and DM spinal cords.

A) Clusterin and B) NSE staining in T12 spinal cord section from archival control. C) Clusterin and D) NSE staining in T12 spinal cord section from archival DM. E) Higher magnification of C) at 20X, taken from ventral horn area, clearly demonstrating the dark, punctate staining pattern in neuronal cytoplasm. F) NSE staining from horse celiac ganglion section shows positive staining in neuronal cell bodies, therefore serves as internal control for NSE. Neuronal cell bodies were marked with arrow (courtesy slide and image from Dr. Pamela Johnston).

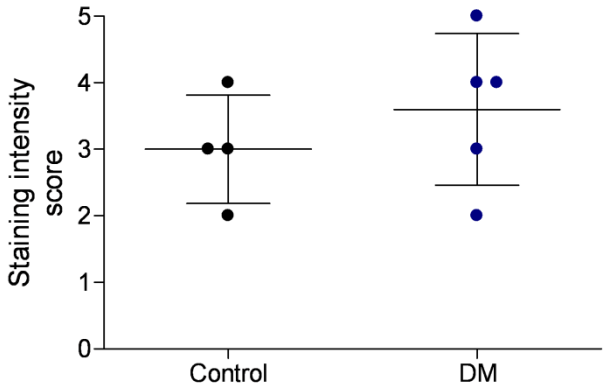

Figure 6-7: The qualitative assessment of clusterin IHC in control and DM cases.

The staining intensity of clusterin in neuronal cell bodies based on a scoring system did not reveal significant difference between control (*N*=4) and DM (*N*=5) groups. Data presented as mean ± standard deviation.

6.5 Discussion

Clusterin is a ubiquitous and highly conserved glycoprotein, expressed by a wide range of tissues and biological fluids (Jones and Jomary, 2002). It is a heavily glycosylated protein, containing six glycosylated sites each of which can bind a variety of ligands, which is the mechanism underpinning the diversity of clusterin in cellular activities (Calero et al. 2005). Clusterin has also been proposed to act as a chaperone molecule involved in the regulation of extracellular protein folding (Nuutinen et al. 2009; Wyatt et al. 2009). In addition, there is strong evidence that clusterin can have a protective role during oxidative stress (Calero et al. 2005; Carnevali et al. 2006). The clusterin protective role is potentially mediated by its chaperone function by facilitating the clearance of misfolded proteins due to the damage induced by altered cellular oxidation status (Poon et al. 2002; Wyatt et al. 2011). Consequently, this may have contributed to the upregulation of clusterin as a response to oxidative damage in DM (Strocchi et al. 2006). However, under chronic stress, it has been reported that clusterin may deviate from its protective function and could potentially promote or enhance protein aggregation (Poon et al. 2002).

In this chapter, we have investigated the potential origin of elevated clusterin in DM CSF (see 5.4.4.3, page 153). Thirty-five to eighty percent of the CSF proteins are blood-derived, and are transported from the blood vessels to the CSF pathways through the blood-CSF-barrier (Reiber and Peter, 2001). Therefore it is tempting to speculate that elevation of clusterin in CSF could be a consequence of clusterin elevation in blood. The protein may enter the systemic circulation then accumulate in the CSF following transport across the blood-CSF-barrier (Figure 6-8A). The characterisation of clusterin by Western blot in this study has confirmed that clusterin is highly abundant in plasma, expressed as the β-chain heterodimeric form with a molecular weight size of 38kDa that is comparable in canine CSF. Clusterin levels in plasma have not been investigated in ALS. However, clusterin elevation in plasma has been reported in AD (Nilselid et al. 2006; Schrijvers et al. 2011), and is described to be associated with the risk, severity and progression in Alzheimer's patients. In this study, the plasma clusterin levels in IE and DM was not significantly different, which makes it unlikely to be the source of elevated clusterin in DM CSF.

An interesting observation of the plasma clusterin is the gel shift detected in 60% (3/5 cases) of DM homozygotes. This observation is suggestive of post-translational modification (PTM). Post-translational modification of proteins are covalent processing

events that can modulate the protein properties, for example by proteolytic cleavage or by the addition or removal of a modifying group to one or more amino acid residues (Seo and Lee, 2004). Under normal physiological conditions these chemical modifications of proteins normally occur after the protein translation step however they may be triggered as a result of pathological processes (Li et al. 2010). The causative mechanisms that lead to a pathogenic PTM is not well understood, however recent evidence has indicated that oxidative stress could induce PTM, which may lead to the alteration of protein function (Trougakos and Gonos, 2009; Kishimoto et al. 2011; Xiang et al. 2013). However, given that the CSF clusterin did not demonstrate PTM, the significance of the clusterin gel shift in plasma samples remains obscure.

It can also be speculated that the elevation of CSF clusterin observed in DM may be due to increased clusterin gene transcription in DM motor neurons in thoracolumbar spinal cord, with a concomitant increase in clusterin protein synthesis, which is potentially secreted and/or translocated from the spinal cord parenchyma into the CSF in the subarachnoid space (Figure 6-8B). The movement of molecules between the spinal cord parenchyma and CSF is complex and remains speculative (Brodbelt and Stoodley, 2007). There is evidence of a potential CSF flow into the spinal cord parenchyma through the Virchow-Robin space, and conversely from the parenchyma into the CSF (Stoodley et al. 1996). This may explain how clusterin from motor neurons can accumulate in the CSF. This outflow mechanism is potentially regulated by glia limitans (Engelhardt, 2010).

mRNA expression is informative in predicting protein expression levels in relation to gene function (Guo et al. 2008). The expression of clusterin mRNA has been described in almost all mammalian tissue (Calero et al. 2005) including CNS (Nuutinen et al. 2009; Charnay et al. 2012) and therefore expression of clusterin mRNA in spinal cord is a reasonable expectation. The quantification of clusterin mRNA in DM spinal cord in this study demonstrated a 42% increment compared to controls, implying that CSF clusterin elevation may be derived from spinal cord parenchyma. There is one report investigating clusterin in ALS, to determine if an inflammatory mechanism contributes to the potential aetiology in sporadic ALS. The quantification of clusterin and C1qB (a complement protein in the inflammatory cascade) mRNA from the frontal cortex of sporadic ALS cases demonstrated 40% elevation in ALS relative to control (Grewal et al. 1999). *In situ* hybridisation also demonstrated that clusterin mRNA was increased in anterior gray horn spinal cord of sporadic ALS patients, an area that is severely affected by

neurodegeneration (Grewal et al. 1999). These findings may suggest the involvement of inflammatory process in sporadic ALS. In contrast, inflammatory mechanisms observed in DM are described as a secondary response to the neurodegenerative process (Johnston et al. 2000; Coates and Wininger, 2010). An alternative proposal of ALS pathomechanisms involves the oxidative stress due to the *SOD*1 mutations, which could lead to the death of motor neurons (Nagai et al. 2001; Rothstein, 2009). Clusterin itself has been implicated in the oxidative stress pathway, which has been described to have a protective role against an abnormal redox environment (Carnevali et al. 2006). Over-expression of clusterin mRNA was detected in neuronal and glial cells from rat brain that had been subjected to oxidative stress (Strocchi et al. 1999; Strocchi et al. 2006). Therefore, it is hypothesised that the elevation of clusterin mRNA in DM may result as a response to oxidative stress.

The characterisation of clusterin expression in neuronal and supporting glial cells has been described in several studies (Harr et al. 1996; Lidstrom et al. 1998; Sasaki et al. 2002; Charnay et al. 2012). In normal CNS tissues clusterin is ubiquitously expressed with strong expression in the pontobulbar and spinal cord motor nuclei, and distributed in the neuronal cytoplasm (Charnay et al. 2012). In addition, strong expression of clusterin has been detected in dorsal root ganglia (Charnay et al. 2012), astrocytes (Charnay et al. 2008) and the ependymal cell layer of the choroid plexus (Aronow et al. 1993). In this study, strong clusterin immuno-reactivity has been detected in the motor neurons of T12 spinal cord in both archival DM and control cases. The signal is characterised by a punctate granular pattern that is mainly localised to the neuronal cytoplasm, which is also consistent with the previous studies (White et al. 2001; Charnay et al. 2012). The subjective assessment of the staining intensity by IHC between archival control and DM groups found no significant difference. These findings are not consistent with the observation of increased clusterin mRNA in T12 spinal cord in DM, however the use of clusterin mRNA expression pattern to predict clusterin protein level is informative but not definitive since alteration of protein level does not always correlate with the mRNA expression (Al-Saktawi et al. 2003). Additionally, it is acknowledged that IHC, particularly when using chromogen detection, is a qualitative but not quantitative analytical technique.

Since it is reasonable to assume that the *Sod*1 mutation results in oxidative stress, the elevated CSF clusterin in DM may be induced as a response to this toxic event. We have investigated a number of possible routes that could account for the elevated CSF clusterin

in DM. The elevation of clusterin in DM CSF may reflect the expression of clusterin in the spinal cord parenchyma. Although increased staining of clusterin protein is not observed in DM spinal cord tissue, clusterin expression may be controlled by complex regulatory mechanisms and therefore may not necessarily correlate with the mRNA expression. In addition, it is also possible that clusterin could be directly secreted into the CSF via ependymal cells of the choroid plexus, perhaps also as a response to oxidative stress in DM. The findings from this study have indicated the potential role of clusterin in DM pathogenesis however this proposal would require further study to investigate this possibility.

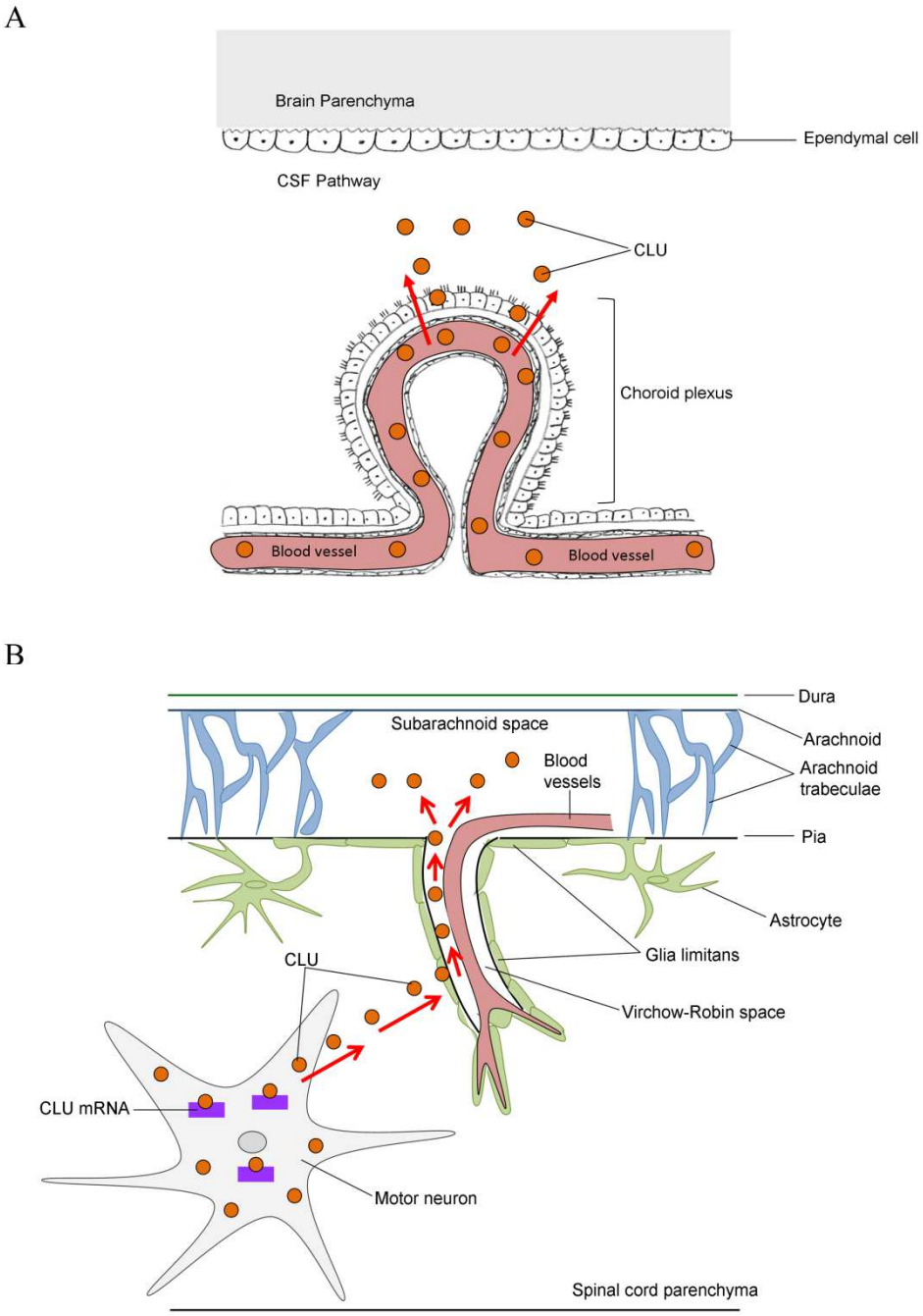

Figure 6-8: The potential underlying mechanisms lead to CSF clusterin elevation in DM

A) Diagram illustrating the blood, CSF and brain interfaces. CSF clusterin elevation may reflect the changes in the blood clusterin levels. This protein may leave the blood vessels and enter the CSF pathways through the tight junctions between the ependymal cells of choroid plexus. B) Compartment model of CSF and spinal cord parenchyma interfaces. Increased clusterin mRNA expression with a concomitant increase of clusterin distribution in DM motor neurons may lead to the CSF clusterin elevation. The potential mechanism involves the movement of clusterin from motor neuron into subarachnoid space via Virchow-Robin space. Clusterin is subsequently disseminated throughout the CSF pathway.

7 General Discussion and Future Directions

7.1 General Discussion

Degenerative myelopathy is a spontaneously occurring, adult-onset, progressive neurodegenerative condition that has been recognised as a clinicopathological entity for many years. The condition is particularly prevalent in GSD, however a number of other specific breeds are also affected (see 1.1.2, page 25). Although the clinical and pathological characteristics are well-defined, the limited understanding of the underlying aetiology as well as the lack of a specific diagnostic test has led to complications in making diagnoses and tailoring management in DM. The confirmation of diagnosis remains at the level of histopathological examination. In addition, the clinical presentation of DM can mimic many acquired spinal cord diseases that may also co-exist with DM, confounding diagnosis. Numerous hypotheses on the potential aetiology have been explored however the high incidence of DM in specific breeds implies a genetic contribution in DM.

The speculation of a genetic basis of DM has recently been substantiated (Awano et al. 2009). A genetic study has established that the occurrence of DM is strongly associated with a mutation in *Sod*1 gene (118G>A or E40K) at the same time implying DM is potentially orthologous to ALS (Ticozzi et al. 2011). The E40K *Sod*1 mutation has been recognised as a major risk factor in developing DM, however it does not appear to be specific to DM as the mutation is also seen in a proportion of the non-affected individuals (Awano et al. 2009). In addition, a recent report has identified a novel *Sod*1 mutation (52A>T) in an affected BMD (Wininger et al. 2011), implying that there is a potential emergence of the new mutation in DM. It is clear that additional indices such as clinical biomarkers are required to specifically differentiate DM from other neurological diseases in the clinic, as well as potentially providing new insights into disease mechanisms. The successful development of DM biomarkers as an adjunct assay that are complementary to genetic marker and current diagnostic methods used in DM would be of substantial value to owners and clinicians.

The main aim of this research is to establish a potential biomarker for DM to facilitate clinical diagnosis. In this study, CSF was selected as an appropriate source for DM biomarker as it is in direct contact with the affected system and can reflect the biochemical changes in any ongoing pathological process of DM. CSF material is also routinely collected for diagnostic purposes, therefore has become a feasible choice for DM biomarker investigations. Since DM has been considered a spontaneously occurring animal

model of ALS, we evaluated selected promising biomarker candidates of ALS in DM CSF; TTR, cystatin C, 7B2 and VGF proteins. This research was also driven by the rapid advancement and the easy accessibility of the proteomic technologies. The 2-DGE protocol was initially considered as a convenient proteomic strategy however optimisation of 2-DGE protocols in canine CSF have failed to achieve acceptable protein separation and good gel resolution. Thus the identification of a novel CSF biomarker in DM was accomplished using a conventional 1-DGE followed by MS techniques, which has led to the significant discovery in this project.

In this study, we have confirmed that canine CSF is a promising source of biomarkers for canine neurological disorders. However, a standardised protocol for CSF handling should be established to minimise the impact of the pre-analytical factors (Ferguson et al. 2007; Teunissen et al. 2011). The relatively distant geographical locations of the small animal hospital and the laboratory have been recognised as having the potential to introduce pre-analytical variables that could compromise biomarker investigation as organisational requirements mean that research staff are not readily able to be present at the time of clinical investigation to manage samples and storage of samples for a period in the clinic is likely to be required. A standard protocol was implemented where the CSF samples were temporarily stored at -20°C (for a maximum 3 days), thawed in ice before centrifugation and then transferred to -80°C for long term storage. This protocol is more convenient in a busy hospital and laboratory with a limited number of technical support staff, however it is strongly suggested that sample transfer to -80ºC should be prioritised whenever possible (Teunissen et al. 2011). Recent evidence has shown that CSF is stable when stored at -20ºC for a short period of time, however storage of CSF at -20ºC for three months and beyond has clearly demonstrated alterations in CSF protein concentrations (eg., cystatin C)(Carrette et al. 2005; Boccio et al. 2006). A potential drawback of our protocol is that the CSF centrifugation step can only be performed after CSF storage at -20°C. CSF centrifugation is strongly recommended prior to first time freezing to prevent the release of cellular proteins due to cell rupture as a result of freeze-thaw cycle that could potentially influence the composition of CSF proteome (Bjerke et al. 2010). It is acknowledged that omitting the centrifugation step prior to -20°C storage may affect the specific CSF protein levels particularly in inflammatory conditions, however since DM and other controls demonstrated relatively low cellular concentrations, we speculated that the impact of cell rupture on the specific CSF protein levels investigated in this study would be minimal.

Another pre-analytical factor that may arise from the clinical or laboratory environment is the sample handling temperature (Schoonenboom et al. 2005; Ferguson et al. 2007). The time delay between the collection procedure and -20ºC or -80ºC storage has been reported as a critical variable affecting biomarker investigations (Ferguson et al. 2007). Thus, the effect of three potential CSF handling practices (4ºC, 37ºC and room temperature) on four specific proteins was conducted in canine CSF. These are all conditions that are recognised in a busy emergency unit and have been investigated to some degree in human CSF (Boccio et al. 2006; Ranganathan et al. 2005; Kaiser et al. 2007). Four candidate proteins were selected based on preliminary characterisation by Western blot and MALDI-TOF MS; TTR, cystatin C, haptoglobin and clusterin. Our findings have confirmed that CSF proteins are differentially affected by CSF handling, specifically dimeric TTR and clusterin levels at 37ºC and room temperature (Appendix 8.5.2). Although CSF proteins have proved to be stable at 4°C overnight we strongly recommend that CSF should be immediately stored at -20ºC or -80ºC, unless examination of CSF cells is required. Immediate CSF centrifugation should also be considered when pursuing biomarker analysis involving samples with a high cellular content (eg., neuroinflammatory cases). Storage at 37ºC and room temperature even for a short period should be avoided however recent reports have demonstrated that room temperature storage for two hours did not alter the protein levels (Teunissen et al. 2011; Vanderstichele et al. 2012). Posting CSF samples is only advised if dry ice is available for packing and should be carried out early in the week to avoid the possibility of delayed storage at -80°C (Teunissen et al. 2009). The findings from this experiment have assisted CSF sample collection in this project, which was achieved through co-ordinated interaction between the clinical and research staff, with a mutual understanding of the significance of the sample handling protocols. Based on the observations we and others have described, a standard unifying procedure for collection and storage of canine CSF for biomarker investigations has been established (Appendix 8.5.3).

The lack of a specific diagnostic test and the paucity of biological material for histopathological confirmation have severely limited the progress of DM research. Prior to the establishment of the genetic basis for DM, the inclusion criteria for DM investigations have relied completely on anamnesis and supportive clinical findings. Use of this potentially heterogeneous population as a basis for the study of DM contributed to the slow progress towards elucidating the aetiology of DM. In this study, we have developed the *Sod*1 genotyping, which has allowed DM stratification on the basis of a specific genotype

and biomarker evaluation in a homogenous group. Heterogeneity in a disease has been recognised as a variable that could complicate biomarker identification (De et al. 2011; Laifenfeld et al. 2012). Stratification of patients into a homogeneous subtype has been a natural strategy for biomarker research and has proved to increase the specificity and reliability of clinical biomarkers (Adewale et al. 2008). In DM the lack of understanding of heterozygous inheritance may introduce a significant variation in the affected group. Therefore heterozygous individuals with a presumptive diagnosis of DM were excluded from further studies at this point.

The establishment of homogeneous control groups with specific genotype could not be accomplished due to limited clinical material and high number of heterozygotes identified in this study. As a result, the control group is a mixture of wild type and heterozygous individuals. This may not be ideal for disease comparison however the heterozygous controls were strictly selected from a dog population that had not shown typical signs and clinical progression of DM. DM is considered as an age-related neurological disorder, therefore ideally cases selected for the control group would be age matched cases as well as genotyped. Inclusion of age matched control cases may enhance the detection of subtle and specific changes between DM and other co-existing disorders at the same time eliminate age as a confounding variable. However, obtaining an age matched control group is a major challenge in clinical research (Hulley et al. 2007). In this study, the establishment of age-matched control group could not be accomplished due to the lack of clinical material from aged dogs presented in the UGSAH, although a small number of cIVDD cases were included in the disease comparison. The lack of age matched controls is also due to the ethical restrictions pertaining to collection of clinical samples from healthy dogs. In this study, an alternative option was adopted by having additional sets of control groups with a younger mean of age such as IE and MEN cases (Appendix 8.5.4). These groups do not present any clinico-pathological features that are associated with DM. IE patients were considered as an ideal set of controls in this study since this group has closest biochemical characteristics to ‘healthy’ individuals, albeit the young age of onset. Correlation analyses were also performed between altered CSF proteins with age, which demonstrated no significant relationships. These analyses are important to determine age-related changes on the specific protein levels since there was lack of age-matched controls for biomarker analyses.

The inclusion of the lumbar CSF samples (*N*=3) in this study has also been identified as a potential variable that may influence the specific protein levels in canine CSF. In this study, the CSF samples obtained from lumbar cistern tend to display higher protein intensities compared to cisterna magna CSF. However, exclusion of these lumbar samples and re-assessment of the statistical analyses did not influence the protein changes in the specific studies.

The characterisation of ALS-associated proteins in DM CSF has proven that these potential ALS biomarkers can be translated to the DM model, although there is a limitation pertaining to the availability of commercial antibodies for canine material. The outcome achieved in this study has provided potential overlapping molecular features between ALS and DM, substantiating DM as a model for ALS. Additionally we have also demonstrated that the application of proteomic technologies, such as MALDI-TOF MS can be optimised in canine CSF, which has facilitated in the identification of a novel biomarker in this project. With the continuing collaboration established in this project and the rapid advancement of proteomic technologies, we contemplate that the biomarker development in DM will become more feasible in future.

CSF biomarker analyses have demonstrated that TTR and clusterin are potential candidates as DM biomarkers. The significant reduction of TTR dimeric levels was observed in DM CSF, which is similarly demonstrated in ALS CSF (Ranganathan et al. 2005; Ryberg et al. 2010). Although this observation was not consistent in the subsequent comparative analysis, further characterisation of TTR in DM may hold great promise, not only as a specific biomarker but may also foster the delineation of the pathogenic pathways in DM. Though a significant clusterin elevation was observed in DM CSF this was not specific to DM as a similar pattern was also detected in cIVDD CSF, although we demonstrated that clusterin is elevated by 20% in DM compared to cIVDD CSF. These findings strongly support the biomarker observations in human that a panel of biomarkers rather than a single biomarker is required to achieve high specificity for diagnosis confirmation (Tainsky, 2009).

The need for a panel of biomarkers in DM also implies the potential involvement of a complex underlying pathogenesis in DM. In *SOD*1-linked ALS, it has been shown that motor neuron death is potentially mediated by oxidative stress through mutation-induced structural changes of SOD1 enzymes (Beckman et al. 1993; Pasinelli and Brown, 2006). The discovery of a *Sod*1 mutation in DM is therefore exciting given the hypothesis of the

oxidative stress in *SOD*1-linked ALS. The consistent presence of SOD1 cytoplasmic inclusion bodies in the spinal cord of *Sod*1-linked DM cases has suggested a possible contribution of oxidative damage in DM pathogenesis. The results on clusterin in this study have provided further evidence supporting the occurrence of oxidative stress leading to motor neuron death in DM. We speculated that the elevation of clusterin in CSF may reflect a response to the oxidative stress event. The secretion of clusterin may be activated directly by ependymal cells of choroid plexus or spinal cord parenchyma to provide protection to neuronal cells against oxidative damage. However, the clusterin function may be impeded or modified during the advanced stage of disease, which could impair the proteosome system and promote protein aggregation that is toxic to motor neurones. The involvement of TTR in the oxidative stress pathway remains unknown, although there is a report that has established a connection of oxidative stress and TTR in Alzheimer's disease (Gustaw et al. 2009). Interaction of clusterin with TTR has also been described through inhibition of TTR-associated amyloid formation by clusterin in TTR amyloidosis (Lee et al. 2009). Therefore, there is a possibility that these two proteins; clusterin and TTR could have a biochemical link in DM pathogenesis. Alternatively, it is also possible that TTR may have a different role in DM pathogenesis.

In conclusion, the realisation of this research has provided a significant contribution to the establishment of potential biomarkers for DM as well as generating evidence on the potential underlying mechanisms in DM. Clusterin and TTR may represent components in a panel of emerging biomarkers that may combine to distinguish DM in the clinic. Although these biomarkers may require an extensive validation process prior to their translation into clinical practice, the successful translation of reliable and effective biomarkers for DM would enhance diagnosis and subsequently address the issue of therapeutic intervention. However, one has to remember that the use of DM biomarkers alone may not be sufficient to provide a specific diagnosis. Therefore, it is also pertinent to propose that the biomarker information is only clinically meaningful when used in conjunction with current diagnostic methods.

7.2 Future Directions

In this dissertation I have shown that canine CSF is an appropriate source of biomarkers for chronic neurodegenerative disorders such as DM, provided that the sample reliability is not compromised by pre-analytical factors. Due to the limited clinical material the

biomarker evaluations of clusterin and TTR in this study were performed on a small DM population. Therefore, further validation of these candidate proteins in a large-scale DM population would be of interest as samples become available. This will be achieved through multi-centred studies using common clinical criteria with mutual agreement of standard sample handling protocols. In addition, since there are no clinical differences observed between *Sod*1 genotypes in affected dogs, it is our intention to evaluate the candidate CSF proteins established from this study in affected individuals with wild type or heterozygous genotypes.

Future work will focus on the characterisation of additional novel candidate proteins (eg., apolipoprotein E) as DM biomarker by proteomic approach. Alternative sources of body fluids such as blood (serum and plasma) or urine will also be considered for future biomarker investigations in DM, although CSF will remain the most preferable source of biomarker. Steps that have been taken to accomplish this objective include the establishment of collaboration with the CSF proteomic experts in the University of Rome, who specialise in the linear model of MALDI-TOF MS. Exchanges of clinical material between groups have been achieved recently and the optimisation of this technique on canine CSF is currently ongoing. Further characterisation of TTR in DM CSF (eg., PTM) will also be accomplished using the linear model MS technique. This experiment could further substantiate the hypothesis of DM as a naturally occurring model of ALS. In addition, the collaboration with the proteomic group in the University of Glasgow has been established to address the issues with 2-DGE protocol in canine CSF.

For several years, our group has been investigating the nature of the pathology in DM. In ALS *SOD*1 mutations have been linked to oxidative stress contributing to motor neuron death, potentially mediated through altered conformation and biochemical properties of SOD1 enzymes. We continue to explore the impact of the *Sod*1 mutation (118G>A) in DM, wether this mutation induces a misfolded sod1 conformation that can subsequently lead to formation of aggregates and disruption of mitochondria. These projects are being undertaken in collaboration with ALS experts; Professor N. Cashman (University of British Columbia) and Professor P.J. Shaw (University of Sheffield). Our findings on clusterin in DM would also appear to support the involvement of oxidative stress in DM. Similar *in vitro* system will be used to investigate the clusterin expression in mutant *Sod*1 transfected cells under oxidative stress condition (e.g., pre-treating cells with hydrogen peroxide).

Further assessment of the potential areas described in this section may facilitate the future development of DM biomarkers and at the same time could assist the biomarker translation into clinical practice. Novel hypotheses on the potential pathogenic mechanisms in DM have also been identified in this project. Further investigation of the questions raised in this study may highlight the commonality between ALS and DM, which will be of mutual benefit to both research communities.

8 Appendices

8.1 List of Cases Included in This Project

8.1.1 CSF Biomarker Study

No	Case ID	Breed	Age (y,m)	Sex	Diag.	*Sod1*	CYS C	TTR		Hp	CLU			Remarks
							4.4.3.1	4.4.3.2	4.4.3.4	5.4.4.1	5.4.4.2	5.4.4.3	6.4.1	
1	213652	GSD	0,7	M	AR. CYST	HET								
2	205302	GSD	8,10	M	DISC	HET								
3	210822	CKCS	9	MN	DISC	HET			●			●		
4	222048	LABRADOR RETRIEVER	8,5	M	DISC	WT			●			●		L (45mg/dl)
5	222730	GSD	6,6	M	DISC	WT	X	X	X	X		X		▲
6	224382	GSD	7,11	F	DISC	HET			●			●		
7	227633	BMD	9,11	MN	DISC	HET			●			●		
8	227843	GOLDEN RETRIEVER	10,5	M	DISC	WT			X			X		Acute disc
9	227934	GSD	8	M	DISC	WT			X			X		▲
10	XN4830	GSD	8	M	DISC	HET	X	X	X	Xs	X	X	X	▲
11	211948	GSD	0,7	M	DM	HET		X		Xs	X		X	
12	212078	GSD	7,4	MN	DM	HOMO	●	●	●	●	●	●	●	

No	Case ID	Breed	Age (y,m)	Sex	Diag.	*Sod1*	CYS C	TTR		Hp	CLU			Remarks
							4.4.3.1	4.4.3.2	4.4.3.4	5.4.4.1	5.4.4.2	5.4.4.3	6.4.1	
13	212265	GSD	9	MN	DM	HOMO			●		●	●	●	L (58mg/dl)
14	213295	GSD	8,4	MN	DM	HET			X		X	X	X	
15	213587	GSD	7	M	DM	HET			X			X	X	
16	213934	GSD	8	FN	DM	HOMO	●	●		X_S	●		●	
17	214784	GSD	9	M	DM	HOMO	●	●		●	●			
18	221994	GSD	7	FN	DM	HOMO	●	●		X	●			L (60mg/dl) *, ■, ♠
19	222937	GSD	6	M	DM	HOMO	●	●	●	●	●	●	●	
20	224053	GSD	13	MN	DM	HOMO	●	●		●				
21	224481	GSD	8,7	FN	DM	HOMO		●						■, ♠
22	227716	BORDER COLLIE	9	MN	DM	HOMO			●	●	●	●	●	■
23	211392	GSD	5	M	IE	WT	●	●		●	●		●	
24	211763	SIBERIAN HUSKY	3,9	MN	IE	WT	●	●	●	●		●		
25	212083	GSD	3,1	F	IE	WT	●	●		●				
26	212405	BOXER	3	M	IE	HET	●	●		●				
27	212451	YORKSHIRE TERRIER	1	M	IE	HET		●	●	●	●	●	●	
28	212588	BOXER	0,6	M	IE	WT	X	X	X	X	X	X	X	Seizure<3d

No	Case ID	Breed	Age (y,m)	Sex	Diag.	*Sod1*	CYS C	TTR		Hp	CLU			Remarks
							4.4.3.1	4.4.3.2	4.4.3.4	5.4.4.1	5.4.4.2	5.4.4.3	6.4.1	
29	212855	GSD	7,4	M	IE	HET	●	●		●	●		●	
30	214514	DOBERMAN	4	M	IE	HET			●		●	●	●	
31	222373	BORDERCOLLIE	2	M	IE	WT	●	●		●	●			
32	222557	GIANT SCHNAUZER	2	M	IE	WT	●	●	●	X_S	●	●	●	
33	224899	COLLIE	8,2	MN	IE	HET			●		●	●	●	
34	227606	X BREED	9	MN	IE	WT		●	●	●	●	●	●	
35	227698	GSD	3,6	MN	IE	WT			●		●	●	●	
36	211490	BOXER	1,3	F	MEN	HET			●			●		
37	212596	BOXER	4,7	MN	MEN	HET			●			●		
38	212999	PUG	1	M	MEN	HET			●			●		
39	214108	MALTESE	5	M	MEN	HET			●			●		
40	214628	BOSTON TERRIER	2	M	MEN	HET			●			●		
41	214854	LABRADOR	5	FN	MEN	HET			●			●		
42	214885	WHWT	8	FN	MEN	WT			●			●		
43	215185	BICHON FRISE	9	MN	MEN	WT			●			●		

Table 8-1: Signalment for all dogs included in CSF biomarker study.

Following abbreviations are used; German Shepherd Dog (GSD), Cavalier King Charles Spaniel (CKCS), Bernese Mountain Dog (BMD), X breed (cross-breed) male (M), female (F), neutered (N), idiopathic epilepsy (IE), degenerative myelopathy (DM), meningitis (MEN), type II disc disease (DISC), wild type or normal *Sod1* gene (WT), heterozygous (HET), homozygous for mutant A allele (HOMO), cystatin C (CYS C), transthyretin (TTR), haptoglobin (Hp), clusterin (CLU), lumbar CSF (L).

Following symbols are used; cases treated with prednisolone at the time CSF was collected (*), diagnosed with mild disc degeneration (■), had been previously diagnosed with DM (▲), diagnosed with mild spondylosis (♠). Cases that marked "X" were excluded from further studies based on the criteria outlined in 4.3.3, page 116. CSF samples marked "X_S" were excluded due to the lack of signal in Western blots.

8.1.2 mRNA and IHC Studies

No.	Case No.	Control/ Affected	Breed	Age	Sex	Diag.	SC Section	*Sod1*	mRNA	IHC
44	129239	CONTROL	FLAT-COATED RETRIEVER	8	FN	MH	T13	WT	●	●
45	8B95	CONTROL	GSD			NND	T13	WT	●	●
46	129238	CONTROL	GSD	8	M	NND	T12	WT	●	●
47	129237	CONTROL	GSD	8	M	MCT	T13	WT	●	●
48	127761	CONTROL	GSD	3		AF	T13	HET		
49	129202	AFFECTED	GSD	10	FN	DM	T12	HOMO	●	●
50	128291	AFFECTED	GSD	8	FN	DM	T12	HOMO	●	●
51	129800	AFFECTED	GSD	9	M	DM	T12	HOMO	●	●
52	129966	AFFECTED	GSD	9	M	DM	T12	HOMO	●	●
53	126438	AFFECTED	GSD	12	FN	DM	T12	HOMO		●

Table 8-2: Signalment for all dogs included in mRNA and immunohistochemistry (IHC) study.
Following abbreviations were used; malignant histiocytosis (MH), non-neurological disorders (NND), mast cell tumour (MCT), anal furunculosis (AF).

8.1.3 Pre-analytical Assessment

No.	Case No.	Breed	Age (y,m)	Sex	Diag.
1	211392	GSD	5	M	IE
2	212083	GSD	3,1	F	IE
3	212364	WEIMARANER	7,4	M	IE
4	220204	X BREED	4	M	IE
5	222373	BORDER COLLIE	2	M	IE
6	222557	GIANT SCHNAUZER	2	M	IE
7	222706	COCKER SPANIEL	0,3	F	IE
8	223485	BORDER TERRIER	6	MN	IE
9	223800	X BREED	4	FN	IE
10	224372	BORDER COLLIE	7,3	MN	IE

Table 8-3: Signalment for IE cases for pre-analytical assessment.

8.2 Protein Analysis

8.2.1 Tissue Homogenisation Buffer

Amount	Chemical
1ml	10% triton X-100
1ml	10X TBS pH7,4
20µl	0.5M EDTA
5µl	1M DTT
5µl	10mg/ml aprotinin
5µl	10mg/ml leupeptin
5µl	10mg/ml trypsin inhibitor
100µl	100mM benzamidine
100µl	250mM sodium orthovanadate
100µl	100mM sodium pyrophosphate
40µl	250mM PMSF

8.2.2 BCA Reagents

Reagent	Chemical
Reagent A	Sodium carbonate
	Sodium bicarbonate
	Bicinchinonic acid
	Sodium tartrate in 0.1M sodium hydroxide
Reagent B	4% cupric sulphate

The mixture ratio of reagent A and B is 50:1.

8.2.3 Sample Denaturation Buffer (3X)

Amount	Chemical
0.187M	Tris pH 6.8
6%	SDS
30%	Glycerol
0.006%	Bromophenol blue
40mM	DTT

8.2.4 Ponceau S

Amount	Chemical
1g	Ponceau S
50ml	Acetic acid

Dissolve in 1l of distilled water.

8.2.5 T-TBS (10X)

Amount	Chemical
60.2g	Tris-base
87.6g	Sodium Chloride (NaCl)
10ml	Tween 20

Dissolve in 700ml distilled water. Adjust pH to 7.4 with 1M hydrochloric acid (HCl) and make the volume up to 1l.

8.2.6 Hand-poured SDS-PAGE Gel

Chemical	12.5%	Stacker (4%)
Acrylamide:bisacrylamide at ratio 37:1	16.6ml	1.76ml
1.5M Tris pH8.8	10.0ml	
0.5M Tris pH6.8		1.64ml
10%SDS	400µl	60µl
10% ammonium persulphate	300µl	150µl
Ultrapure water	12.8ml	8.0ml
TEMED	20µl	18µl

Volumes for 20 well, large format 1-DGE (16cm x 13cm) with 4% stacker.

8.2.7 Towbin Transfer Buffer

8.2.7.1 Anode 1

Amount	Chemical
36g	Tris
74ml	Methanol

8.2.7.2 Anode 2

Amount	Chemical
3g	Tris
75ml	Methanol

8.2.7.3 Cathode

Amount	Chemical
3g	Glycine
3g	Tris
74ml	Methanol

For all buffers, make up until 1l with distilled water and stored at 4°C.

8.2.8 SDS-PAGE Running Buffer for Hand-poured Gel (10X)

Amount	Chemical
144g	Glycine
30.3g	Tris
10g	SDS

Add the glycine to 700ml of distilled water, once dissolved add Tris and SDS and finally made up the volume of 1l with distilled water.

8.3 Nucleic Acid Analysis

8.3.1 Tris-acetate-EDTA buffer (1X TAE)

Amount	Chemical
4.8g	Tris-base
1.1ml	Acetic acid
2ml	0.5M EDTA

Volume made up to 1l.

8.3.2 Tris-borate-EDTA (1X TBE)

Amount	Chemical
10.8g	Tris-base
5.5g	Boric acid
2ml	0.5M EDTA

Add the following reagents into 800ml of distilled water and adjust to make up final volume of 1l.

8.4 Tissue Morphological Analysis

8.4.1 Buffered Neutral Formaldehyde (4%)

Amount	Chemical
100ml	40% formaldehyde
900ml	Tap water
4g	Sodium dihydrogen orthophosphate
8g	Dipotassium hydrogen orthophosphate

8.4.2 Dewaxing and Dehydrating Sections

Step	Reagent	Duration
Dewaxing	Histoclear	2 minutes
	70% alcohol	2 minutes
	70% alcohol	2 minutes
	70% methylated spirit	2 minutes
	Water	5 minutes
Dehydrating	70% methylated spirit	2 minutes
	70% alcohol	2 minutes
	70% alcohol	2 minutes
	Histoclear	10 seconds
	Histoclear	10 seconds
	Histoclear	10 seconds

8.4.3 Processing Schedule for Nervous Tissue

Programme 1 : Brain
Duration : 44 hours

Station	Content	Duration
1	70% alcohol	2 hours
2	95% alcohol	3 hours
3	Absolute alcohol	3 hours
4	Absolute alcohol	3 hours
5	Absolute alcohol	5 hours
6	Absolute alcohol	5 hours
7	Absolute alcohol	2 hours
8	Absolute alcohol/xylene	4 hours
9	Xylene	2 hours
10	Xylene	4 hours
11	Wax	2 hours
12	Wax	3 hours
13	Wax	3 hours
14	Wax	4 hours

8.4.4 Scott's Tap Water Substitute (STWS)

Amount	Chemical
8.25g	Sodium bicarbonate
50g	Magnesium sulphate
2500ml	Tap water
2 crystals	Thymol

Total volume for 2.5l.

8.4.5 Sodium Citrate Buffer pH6.0

Amount	Chemical
2.94g	Tri-sodium citrate
1000ml	Distilled water

Total volume for 1l. Adjust to pH6.0 with 1M HCl

8.4.6 1X TBS

Amount	Chemical
12.2g	Tris-base
8.7.6g	NaCl

Dissolve Tris and NaCl in 800 ml ddH_2O. Adjust pH to 7.5 with 1 M HCl and make volume up to 1lwith ddH_2O.

8.5 Additional Information

8.5.1 Two-DGE Analyses in Canine CSF and Brain Tissue

The application of 2-DGE to resolve the proteome of canine CSF was explored in tandem with a dog brain homogenate sample. The resolution of canine CSF by this method can be difficult to achieve due to the high salt content and the presence of high abundant proteins such as albumin. In an attempt to overcome these technical issues, CSF samples were acetone precipitated to remove the salt content and the results are shown in Fig 8.1 (A and B). The while the resolution obtained for the brain sample was satisfactory, the profile obtained with CSF was poor showing a horizontal streak within the area corresponding to albumin. The impact of removing albumin from using the commercially available kit ProteoExtract was investigated by 1-DGE and found to significantly reducte the overall protein content without selectively enhancing the less abundant proteins. A further attempt to selectively reduce albumin and enrich the lower molecular weight proteins and peptides was investigated using a combination of ProteoExtract and the ZipTip C18 resin which can desalt samples and enrich proteins and peptides that are less than 50kDa. 1-DGE demonstrated that while the overall protein content was diminished, there was no significant enrichment in the lower molecular weight proteins. Based on the poor resolution by 2-DGE and the low recovery of less abundant proteins using albumin depletion reagents, it was concluded that this approach would be unlikely to yield reliable and repeatable information of potential biomarkers in CSF.

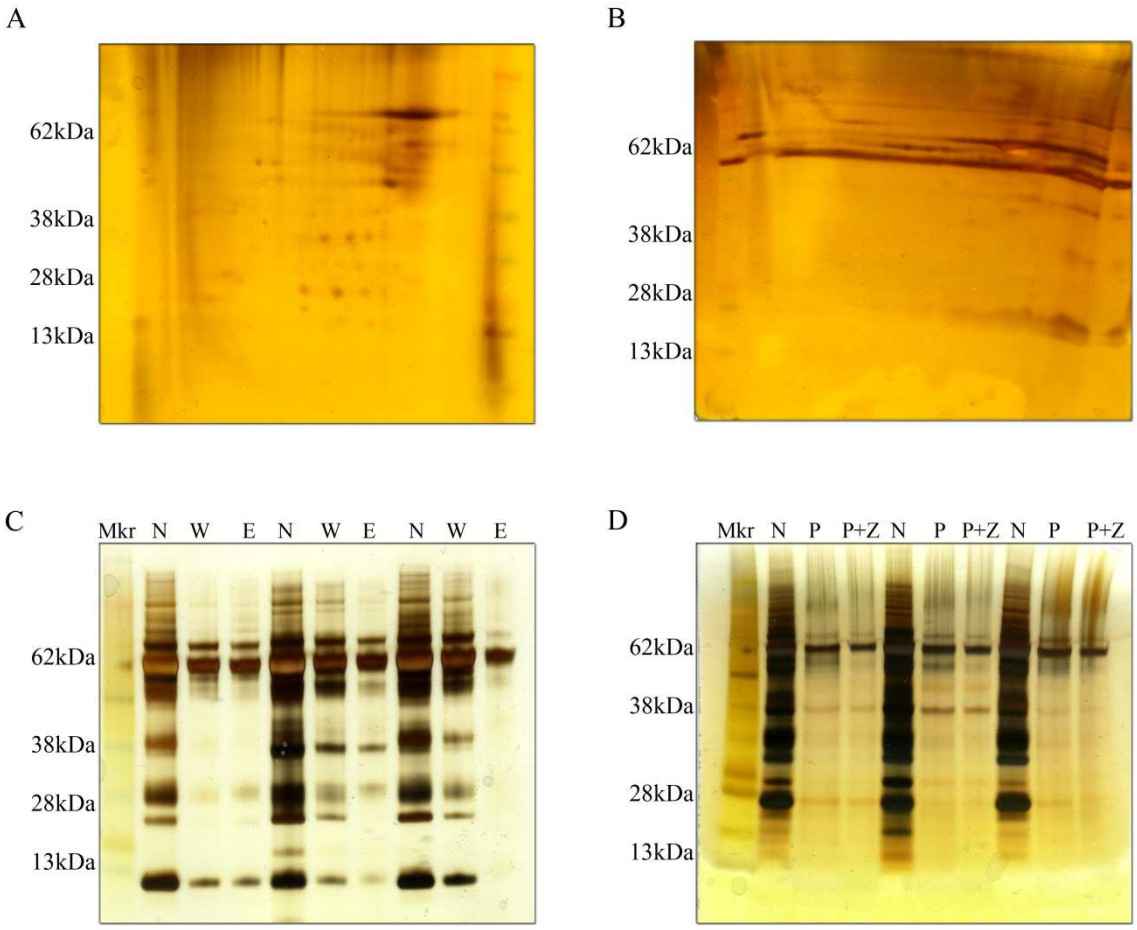

Figure 8-1: Attempts to optimise the 2-DGE protocols in canine CSF and canine brain tissue homogenates.

2-DGE analsysis of actetone precipitated brain extract displayed a sharp resolution of several protein spots across the IEF with pH range of 3-11 (A) while CSF appeared as unresolved horizontal smear (B). Albumin depletion of CSF using ProteoExtract (B) reduced the global protein profile (N) but there was no significant difference between the column wash (W) and the eluted fraction (E). The eluted fraction was also subjected to ZipTip purification (P+Z) but there did not appear to be difference in the profiles before and after ZipTip treatment.

8.5.2 Pre-Analytical Assessment: Summary of Findings

	CLU	Hp	Cystatin C	TTR dimer	TTR monomer
4 °C for 18 hours	-	-	-	-	-
37 °C for 4 hours	-	-	-	50% ↑(**)	-
RT for 48 hours	53% ↓(*)	-	-	40% ↑(**)	-

Table 8-4: The tabulated result of selected CSF proteins stability in canine CSF

8.5.3 Recommendations for CSF Collection for Biomarker Study

Item No.	Procedure	Ideal Situation/Recommendations
1	Site of sampling Cisterna magna vs. lumbar cistern	▪ Either route is preferable, but ideally, analysis should be done using samples collected from one site of sampling ▪ If this is not possible, the gradient effect of protein between cisterna magna and lumbar cistern must be taken into consideration.
2	CSF handling	
	Preferred volume	▪ 1ml for sample banking. Always paired with blood, serum or urine samples.
	Blood contamination	▪ Excluded if visible or exceeding 500RBC/µl
	Collection tube	▪ Propylene tube without anticoagulant
	Documentation	▪ Date of collection, case ID, site of sampling, visible blood contamination or RBC count, time delay between collection and freezing, drugs administration, eg., prednisolone.
	Temporary storage • 4°C • -20°C • Room temperature	 ▪ Not recommended ▪ Less than 3 months ▪ Not recommended
	Transport conditions	▪ In dry ice during early week
3	CSF processing	
	Centrifugation	▪ 1000rpm for 10 minutes ideally at 4°C however at room temperature is acceptable.
	Aliquots	▪ A minimum of two aliquots, and split between -80°C freezers (if applicable)
	Freeze-thaw cycles	▪ Limit the repeated cycles to 1-2 cycles

8.5.4 The Age Comparison in DM and Control Groups in CSF Biomarker Studies

Group	M±SD in years	*N*
IE	4.3±2.6	12
DM	8.5±1.9	14
MEN	4.1±3.1	8
cIVDD	8.8±0.8	4

Table 8-5: The age comparison in DM and control groups in CSF biomarker studies.

8.5.5 Owner's Consent Form

Small Animal Hospital
Department of Veterinary Clinical Studies
University of Glasgow Veterinary School
Bearsden Road, Bearsden, Glasgow G61 1QH
Telephone: 0141 330 5848 Fax: 0141 330 3663
E-mail (for appointments): clinoffice@vet.gla.ac.uk

UNIVERSITY *of* GLASGOW

CONSENT FORM

Case Number:	**Owner:** .
Animal Name:	**Address:**
Age:	
Species/Breed:	
Sex:	
Date:	

I, the owner or agent of the above animal, hereby request that this animal receives such examination and treatment as may be required. This may include the administration of anaesthetic drugs, the act of surgery and treatment with drugs that may only be licensed for use in other species. The nature and effect of this examination and treatment have been explained to me. No assurance has been given that the treatment will be carried out by a particular veterinary surgeon. I understand that students, acting under appropriate supervision, may be involved in the examination and care of my animal. I give my permission for any data or samples obtained as part of the examination or treatment to be retained for further study.

Signed.....

Estimate £ (inc VAT)

I also realise that the estimated costs of treatment can only be approximate and do not include any emergency procedures, follow up treatment or investigation. I accept that I am liable for all costs incurred in the treatment of my animal and agree to pay in full upon collection of my animal.

Signed.....

I confirm that I have explained to the owner or agent the nature and effects of the examination and treatment to be carried out on the above named animal.

Signed.....(MRCVS / VN)

Small Animal Hospital
University of Glasgow Veterinary School, Bearsden Road, Bearsden, Glasgow G61 1QH
Telephone: 0141-330 5848 *Fax:* 0141-330 3663 *E-mail*: clinoff@vet.gla.ac.uk

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