**The impact of aging and the associated inflammatory response in the spinal cord.**

A thesis submitted in partial fulfilment of the

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**ABSTRACT**

Background: The neuroinflammatory response of the body is a critical element of many spinal cord diseases, and understanding the impact of age and sex on the development of neuroinflammation in a non-disease state is an important element of the development of animal models of disease. Astrocytes are the most abundant cell type in the spinal cord, and they perform several immune functions, however their ability to function appropriately is impacted by the effects of age. Methods: Male and Female sheep aged 1-2 (n=16), 3-4 (n=16), and 5-6 years (n=10) were saline perfused, spinal cord extracted and immerse fixed before being prepared for histological processing. Spinal cord sections from three levels (C2, C4, and T2) were prepared and stained for GFAP. Digital images of slides were analysed for GFAP positive astrocytes. Results: No significant differences in GFAP immunoreactivity was observed between age or sex. There was a significant decrease ($p<0.05$) in GFAP positive astrocytes at the C2 level compared to the C4 or T2 levels. Significant differences were also observed between regions of the spinal cord, with greater GFAP immunoreactivity within the dorsal and ventral horns compared to the white matter regions. Conclusion: This study found no significant effect of age or sex, however significant differences in neuroinflammation were seen between levels and regions of the spinal cord. This understanding of neuroinflammation in the non-disease state of the spinal cord will inform the development of animal models of human spinal cord disease.

*241 words*

## INTRODUCTION

Numerous pathologies that affect the spinal cord are associated with an inflammatory response which can increase the level of damage to the spinal cord. Given that these diseases can occur throughout life, it is important to understand how aging itself effects levels of inflammation within the spinal cord and thus may influence the progression of the disease. Disorders that involve inflammation within the spinal cord such as acute transverse myelitis, multiple sclerosis, spinal cord injury (SCI), Brown-Sequard syndrome, arachnoiditis, cauda equina syndrome, and Guillain-Barre syndrome are particularly debilitating as they involve damage to the central nervous system (CNS), and the loss or deterioration of neurological control over the body. The investigation of neuroinflammatory levels in a non-disease state will inform the development of an accurate model of disease that is clinically applicable to humans, as the baseline will be better understood.

Due to the critical nature of neuroinflammation on disorders of the spinal cord, changes to the body's immune responses will impact the progression of the inflammation. Therefore, age may be important to consider, given that the immune response of the body changes with age. This phenomenon has been termed "inflamm-aging" [1], which describes the progression of the body's immune system to a chronic mild pro-inflammatory state, increasing cellular stress and inducing stress response pathways[2, 3]. When immunosenescence (age-related immune alterations) occurs in the CNS, senescent cells express a "senescence-associated secretory phonotype"[3] (SASP) which increases the secretion of pro-inflammatory cytokines, furthering the chronic inflammatory state[3-5]. With increasing age, the spinal cord displays increased apoptosis, an increase in neuronal cell body size, axonal loss and swelling, higher blood spinal cord barrier (BSCB) permeability, and overall decreased neuronal health and integrity[6, 7]. Furthermore, with increasing age, pro-inflammatory intracellular pathways such as nuclear factor kappa B (NFκB) are activated in immune cells of the brain, which modulates the main immune-related gene expression changes, and also provides a direct link between aging and inflammation[5]. Distinct differences have been shown to exist between mature and developing spinal cords in pre-clinical studies where the inflammatory response differed

in profile and timeframe, in both the cellular and molecular response[8]. For example, microglia are activated within 24 hours in all cases of SCI, however young mammals present a markedly less pronounced reaction compared with their adult counterparts[8]. Currently inflamm-aging has been primarily characterised in the brain, however there is some evidence to suggest a similar process occurs in the spinal cord at the cellular and molecular level[6, 9, 10].

Research to date focused on inflamm-aging has highlighted the role of microglia in inflamm-aging in the CNS[1, 5, 11-14]. The changes to microglial function is an accurate marker of inflamm-aging due to their susceptibility to damage caused by age, stress or injury owing to their long lifespan and limited turnover capacity[15]. With increasing age, microglial cell size and granularity increases, as well as displaying an altered morphological profile to a more activated state, heightening the inflammatory status of the aged CNS[7, 15]. This altered morphological profile causes an inability to produce a functional immune response, and instead microglia will produce an exaggerated pro-inflammatory response[13]. The critical roles of microglia has been thoroughly researched in the brain, however the role of microglia and other glial cells essential to the inflammatory response, such as astrocytes, need to be investigated further in the spinal cord to provide information to inform development of models of disease for human spinal cord diseases.

Astrocytes are a critical element of inflamm-aging as they have several immune functions, including possessing several pattern-recognition receptors, as well as secreting cytokines and chemokines[3, 16]. The innate immunity capacity of astrocytes can be activated by a variety of insults, and so astrocytic activation is common in the CNS with disease or injury[3, 16, 17]. Astrocytes in the aging brain display a pro-inflammatory phenotype and secrete cytokines which activate inflammatory neurodegeneration by triggering oxidative stress, and reactive oxygen species-mediated dysfunction in mitochondria and the endoplasmic reticulum[3, 18, 19]. In this way, astroglial senescence likely has more profound effects in CNS pathobiology than microglial senescence as the role and function of astrocytes in maintaining CNS homeostasis is so varied[3]. Astrocytes perform a variety of functions in both physiological and pathological conditions, particularly in maintaining

the metabolic and ionic homeostasis of neuronal cells, structural stability, myelin maintenance, and maintenance of the BSCB and blood-brain barrier (BBB)[3, 20, 21]. It is due to these critical roles that the aging of astrocytes in the CNS is so notable and merits further investigation.

The aging process is linked to an accumulation of nuclear DNA damage caused by a reduced capacity for DNA repair, thus it is particularly noticeable in neurons due to the limited cell proliferation[3, 22-24]. Many of the observed characteristics of the aging brain are functions that are regulated by astrocytes (such as synaptic plasticity and metabolic balance), and yet age-related changes in astrocytes have received much less attention than neuronal alterations[3]. A rodent study of the brain by Campuzano *et al* saw clear indications that astrocytes express a pro-inflammatory phenotype in the CNS through the process of aging[3, 25]. Additionally, Pertusa *et al* demonstrated that astrocytes in a long-term cell culture lost neuroprotective capacity, which indicate that, at least *in vitro*, astrocytes can initiate cellular senescence programs that triggers the pro-inflammatory response[3, 26]. It has also been observed that with increasing age, an accumulation of iron can occur in perivascular astrocytes which enhances BSCB and BBB disruption[3, 27]. Glial fibrillary acidic protein (GFAP) is a specific marker protein of activated astrocytes in the CNS[3]. The expression of GFAP is indicative of inflammation, hence an increase in GFAP is the most common change observed in astrocytes with aging[3, 28-31]. Thus, it has been shown that the functional immune response capabilities of astrocytes are depleted with age, which can be measured by changes in GFAP positive cells with neuroinflammation and age.

In addition to age, studies have shown that neuroinflammation can also vary in a sex-dependant manner as the expression of key inflammatory genes for microglial activation display sexual dimorphisms, as well as changing with increasing age[32, 33]. This has been shown in several rodent studies of inflammation in the spinal cord, which highlight the differences in the regulation of pro- and anti-inflammatory gene expression between sexes, which causes higher microglial activation and increased cytokine production in females compared to age-matched males[34-37]. Additionally, the expression of a number of the genes involved in neuroinflammation are influenced

by the activation of oestrogen receptors in neurons, which is linked to the known stronger inflammatory responses in females and generally more pronounced inflamm-aging in females than males[32, 34, 38]. Given the delicate interactions between the nervous system, the inflammatory response and the impact of age and sex, it is important to utilise an appropriate research model to gain an accurate understanding of these processes relative to the same responses in humans.

Studies on neurological conditions require particular consideration as to the model of disease to be used. As this study will investigate the non-disease state of the CNS, it is pertinent to choose an animal model that will provide results that are translatable to human neurological pathophysiology. Although there are many advantages of using rodents as an animal model of disease, in the case of the CNS, it is appropriate to consider the use of large animal models. The use of large animal models is particularly important for studies of the brain due to the structural similarity of the large gyrencephalic brains to human brains[39, 40]. The size of the brain and spinal cord and relative matter ratios of large animals are more comparable to humans, as well as the ability to utilise clinical monitoring equipment and magnetic resonance imaging during the study[41]. It is also necessary to consider the similarity of the brain to humans when studying the spinal cord, due to the systemic nature of inflammation in the CNS and the fact that damage to one element of the CNS can cause changes in another, influencing the overall response of the immune system[42]. It is also known that the anatomy of the rodent spinal cord differs significantly from that of the human, particularly the location of the cortical spinal tracts, which significantly limits the translatability of results[43]. Thus, this study utilised an ovine model which has a similar brain structure and organisation of spinal tracts to determine the non-disease inflammatory state of the spinal cord with age.

As such, this project sought to address the gap in knowledge associated with the effect of age and sex on neuroinflammation within the spinal cord in an ovine model. It is hypothesised that greater neuroinflammation will be observed in the spinal cords of older compared to younger sheep and females compared to males, as measured by the number of activated astrocytes. This project

aimed to (1) characterise the age-related alterations in the neuroinflammation of the spinal cord of an ovine model using immunohistochemistry (IHC), and (2) investigate the difference in age-related changes in neuroinflammation between sexes in an ovine model, to gain an understanding of these changes in a non-disease state to inform the development of an appropriate model of disease similar to humans.

## MATERIALS AND METHODS

### *Animals*

The use of animals in this study was approved by the animal ethics committees of the South Australian Health and Medical Research Institute (SAM402.19) and complied with the National Health and Medical Research Council code for the care and use of animals for scientific purposes (8th edition, 2013). The study design involved using animals of three age groups, and both ewes and wethers as shown in Figure 1.

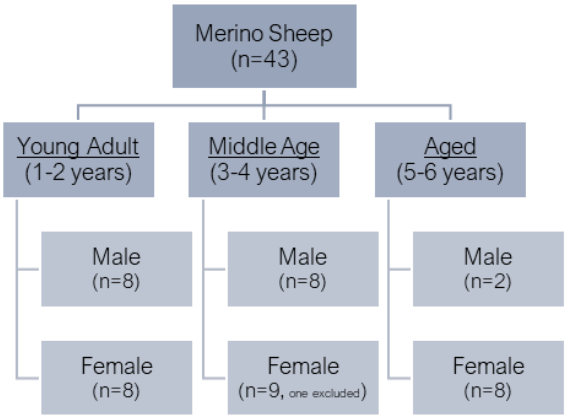


***Figure 1:*** *Study design and animal allocation to three age groups.*

### *Animal perfusion*

Animals were induced with ketamine (5mg/kg; Ceva Animal Health Pty Ltd) and diazepam (Pamlin Injection; 0.1mg/kg; Ceva Animal Health Pty Ltd). The animal was then intubated and anaesthesia maintained with a 3-5% isoflurane with a 100% oxygen mixture administered via an endotracheal tube.

Once deeply anesthetised and shorn, the animal was placed in a supine position and a midline incision made on the ventral surface of the neck. Common carotid arteries were exposed and string loosely tied at both proximal and distal ends of the artery. This was repeated on the second carotid artery. 10mL of heparinised saline solution (25,000 UI) was administered through the jugular vein. Following a 10-minute wait time, the proximal string was tied, occluding the vessel. A small incision was made in the vessel rostrally, and a catheter attached to a perfusion pump was inserted, with the proximal string tied to keep the catheter in place. This procedure was

repeated on the remaining artery. Driving pressure was applied to the perfusion pump, which delivered 10L of tris-buffered saline through the animal's brain vasculature. Incisions in the jugular veins were made bilaterally, allowing perfusate to drain from the animal. After the animal was declared dead, indicated by white gums, the intubation equipment was removed.

***Spinal cord extraction***

The animal was moved to a prone position to allow for spinal cord extraction. An incision was made through the skin, down the entire midline of the spine. After folding the skin back to reveal the muscle mass (see Figure 2A), two parallel incisions were made either side of the spine and the muscle tissue removed to expose the spinous processes (see Figure 2B). The spinous processes were removed, and an oscillating saw fitted with a circular blade was used to saw longitudinally between the lateral surface of the spinous processes and medial to the articular processes, see Figure 2C. The spinal cord was then exposed, and a scalpel used to separate the spinal cord from the spinal nerves and other connective tissue. To prevent the tissue drying out, saline was applied during the process. Starting from the most cranial point available, the spinal cord was gently lifted to view the vertebrae, returned to the canal, and a lateral incision made across the width of the cord in line with the intervertebral disk, see Figure 2D. As the spinal cord was extracted in anatomical level sections, it was placed in cassettes and immersed in 10% buffered formalin.

***Tissue fixing and cutting***

The entire lengths of the spinal cords were fixed, processed, and embedded into paraffin wax in approximately 1cm lengths, with the face of the block being the coronal end of the section. The cervical and upper thoracic regions of the spinal cord – specifically C2, C4 and T2– comprised the focus of the analysis and thus 5μm cross sectional slices were cut using a Leica microtome and placed on slides for immunohistochemistry (IHC).

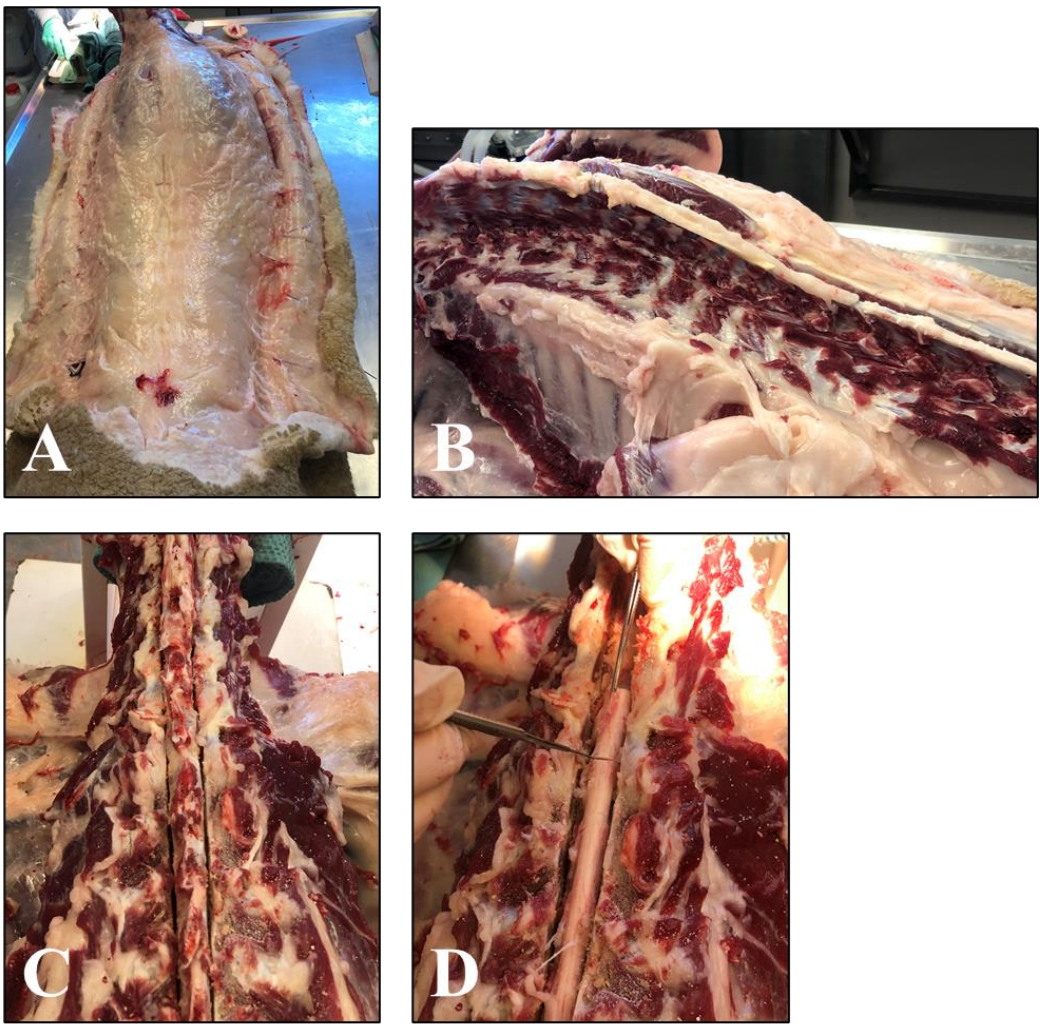


***Figure 2:*** *Images of spinal cord extraction procedure, specifically* ***(A)*** *removing skin,* ***(B)*** *removing muscle to expose spine,* ***(C)*** *sawing either side of spinous processes, and* ***(D)*** *cutting and removing sections of the spinal cord.*

***Immunohistochemistry***

For IHC, slides were dewaxed and immersed in a 1.5% hydrogen peroxide/methanol solution to block endogenous peroxidase activity and washed in phosphate-buffered solution (PBS). Heat-induced epitope retrieval was done with a citrate buffer to recover antigen reactivity. Blocking occurred with 3% normal horse serum (NHS; Vector Laboratories) in PBS. Slides were incubated overnight at room temperature in primary antibody GFAP (1:100,000; Dako #Z0334). Following washing slides had biotinylated horse anti-rabbit IgG (1:250; Vector laboratories; Cat No. BA-1100) applied as a secondary for 30 minutes at room temperature. Finally, a tertiary antibody was applied following washing for 60 minutes in horseradish streptavidin peroxidase conjugate (1:1000;

Vector laboratories; Cat No. SA-5004), and then washed again. 3,3-Diaminobenzidine (DAB) solution was then applied for 7 minutes before the slides were washed in running water. Finally, the slides were counterstained with haematoxylin and cover-slipped using DePex glue.

The tissue sections were digitally scanned using a NanoZoomer (2.0-HT), to be viewed on Hamamatsu NDP.view 2 (v2.7.52). Regions of interest (shown in figure 3) were identified, and representative images for each region captured and exported to Fiji ImageJ (v1.53c). Due to differences in size and shape of regions, the number and magnification of images were specific to each region, as shown in Table 1. Images were processed through a macro to detect GFAP positive (GFAP+ve) cells with a set threshold and particle size, as per Table 1.

| Regions | Number of images per region | Magnification | Threshold | Min particle size (pixels) |
|---|---|---|---|---|
| 3-6 | 4 | 40 | 130 | 240 |
| 1-2 | 2 | 40 | 130 | 240 |
| 7-12 | 3 | 20 | 100/160 | 150/175 |

***Table 1:*** *Image number, magnification, and processing values for ImageJ per region*

Due to differences in the strength of staining, the majority of the images were analysed with one set threshold, however some images within the white matter (regions 7-12) showed a total cell count of 0, where cells could be visually observed in these images (shown in Figure 4). These images were separated and utilised a different threshold and minimum particle size to ensure that the GFAP+ve cells were detected as per Table 1.


***Figure 3:*** *Regions of interest: (1-2) intermediate grey region, (3-4) dorsal horn, (5-6) ventral horn, (7-8) dorsal column, (9-10) lateral column, and (11-12) ventral column.*

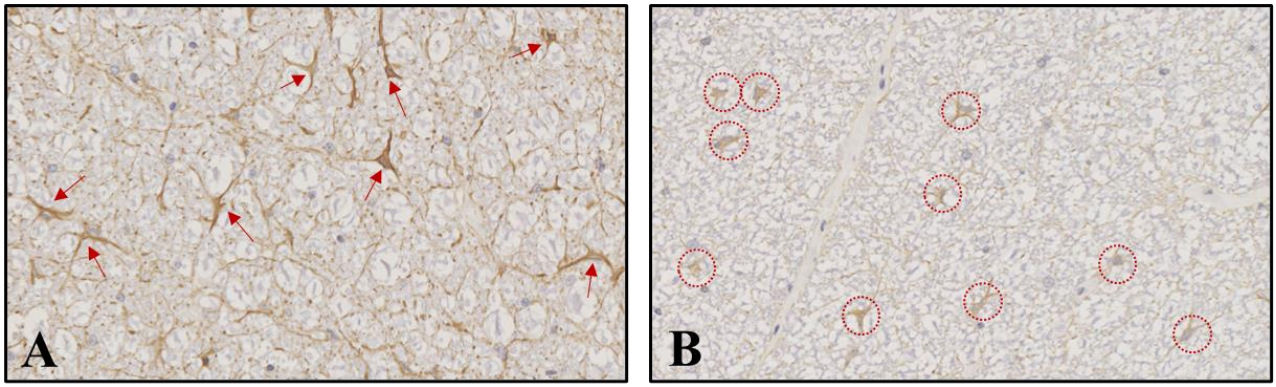


***Figure 4:*** *Differences in strength of GFAP staining* ***(A)*** *shows clear staining, analysed using initial thresholding values, and* ***(B)*** *showing fainter staining which was separated and analysed using the second set of thresholding values.*

***Statistical analysis***

The data produced by ImageJ was the number of GFAP positive astrocytes in each representative image. This data was analysed using a mixed factorial ANOVA with age, sex, region, and level of spinal cord as the four main effects (SPSS, IBM). The Bonferroni *post-hoc* test was used to analyse the main effects or interactions that showed significance. Data is presented as mean ± standard error of the mean (SEM) and a result of significance where the p value is less than 0.05.

## RESULTS

### *Age and sex*

The mixed factorial ANOVA showed no significance in the main effects of age ($F(2,32) = 0.3696$, $p=0.697$) or sex ($F(1,32) = 0.950$, $p=0.337$), shown in Figure 5.

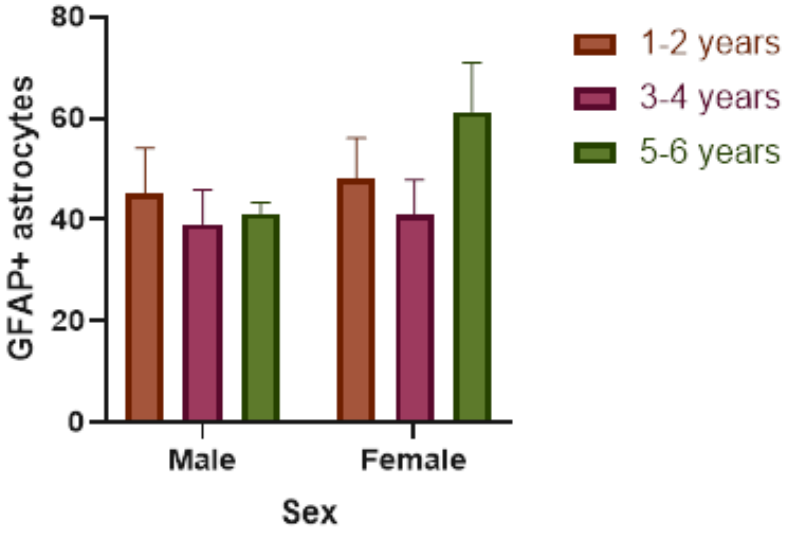


***Figure 5:*** *Histogram of the mean number of GFAP positive astrocytes per age group, by sex of the animal. Using multivariate ANOVA there was no significant difference in either main effect (P>0.05).*

### *Level*

Statistical analysis demonstrated a significant main effect of level ($F(2,64) = 11.987$, $p=<0.001$) and upon Bonferroni *post-hoc* analysis, the average astrocyte number at level C2 (34±3.7 GFAP+ cells) was shown to be significantly less than C4 (52±5.4 GFAP+ cells, $p<0.001$) and T2 (54±5.3 GFAP+ cells, $p=0.001$), as shown in Figure 6A.

### *Region*

There was also a significant main effect of region ($F(5,106) = 17.99$, $p<0.001$). The *post-hoc* test indicated the average number of grey matter astrocytes in the dorsal horn (68±6.7 GFAP+ cells) and the ventral horn (62±7.3 GFAP+ cells) is significantly higher than all white matter regions, as shown in Figure 6B. Within the white matter, the number of astrocytes in the lateral column (29±1.8 GFAP+ cells) is significantly lower than the ventral column (38±2.8 GFAP+ cells,

p<0.001). There was no significant difference in astrocyte number between regions within the grey matter. Figure 7 shows representative images of the specific grey and white matter regions.

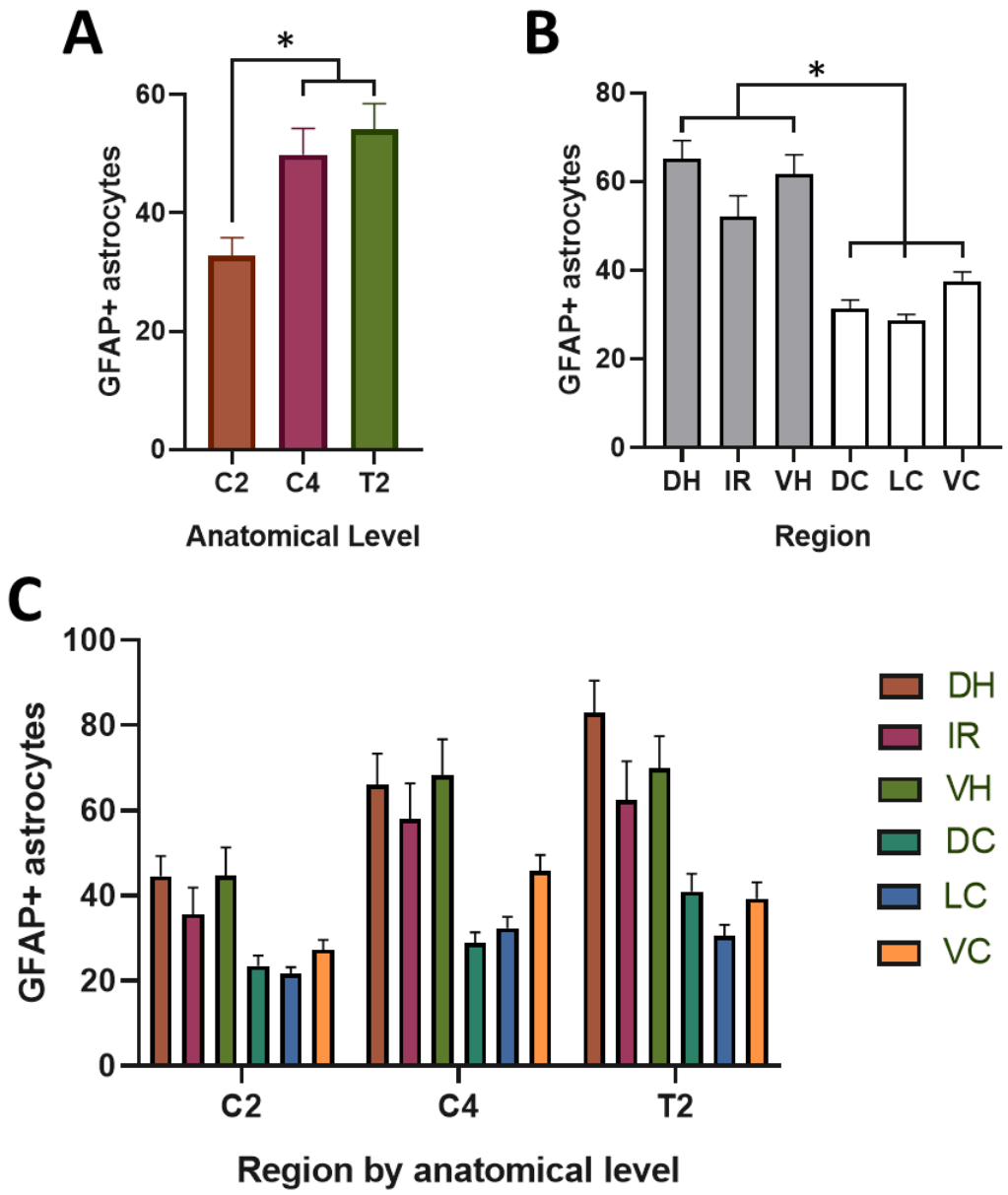


***Figure 6: (A)*** *The number of GFAP positive astrocytes at each anatomical level of the spinal cord. Using Bonferroni post-hoc test, a significant difference ($p \leq 0.001$) was found between the number of astrocytes at C2 and those at C4 and T2.* ***(B)*** *Number of GFAP positive astrocytes in each region of interest. DH = dorsal horn, IR = intermediate region, VH = ventral horn, DC = dorsal column, LC = lateral column, and VC = ventral column. Bonferroni post-hoc test showed a significant difference between: DH and DC, LC, and VC; and VH and DC, LC, VC.* ***(C)*** *Histogram of GFAP positive astrocytes in each region of interest, by anatomical level. * = $p<0.05$; mean ±SEM*

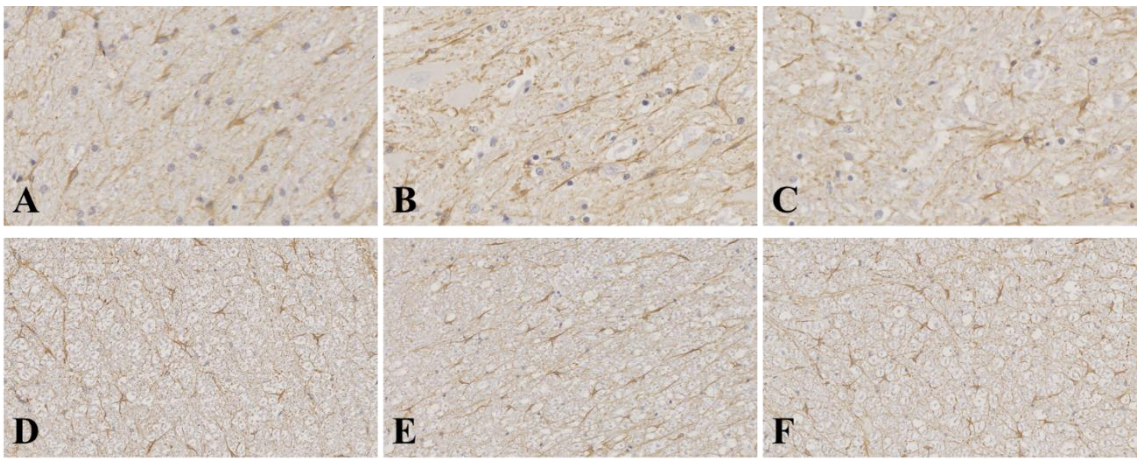


***Figure 7:*** *Representative images of* ***(A)*** *DH,* ***(B)*** *IR,* ***(C)*** *VH,* ***(D)*** *DC,* ***(E)*** *LC, and* ***(F)*** *VC.*

***Level and region interaction***

A significant interaction was found between level and region (F (10,320) =2.1, p=0.024) indicating that there is a significant difference in the pattern of regional difference in astrocytes differed as a function of level of the spinal cord, which can be seen in Figure 6C.

***Level, region and sex interaction***

A three-way interaction was found between region, level, and sex (F (10,320) =1.813, p=0.057) which approached, but did not reach statistical significance, see Figure 8. This indicated that there may be a trend in the data that the way that the level of the spinal cord affects the regional differences differs between males and females.

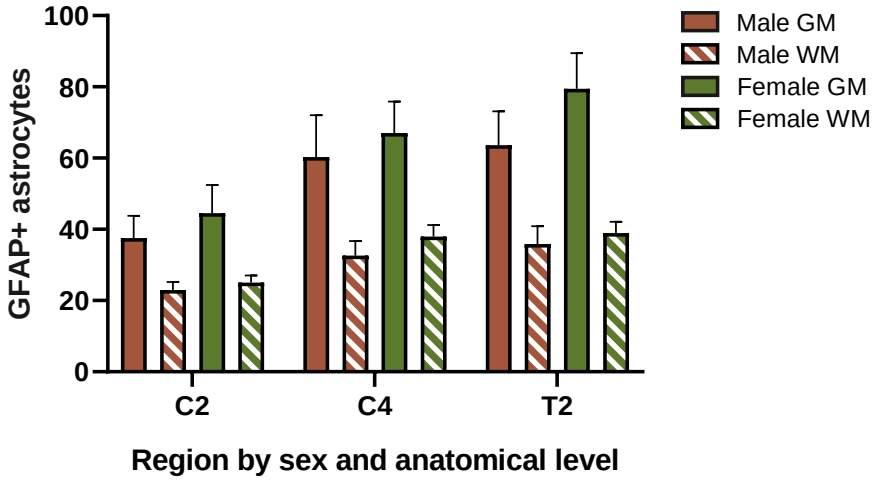


***Figure 8:*** *Histogram of the mean number of GFAP positive astrocytes by matter type, anatomical level and sex. Using multivariate ANOVA there was differences approaching but not reaching statistical significance (p=0.057).*

## DISCUSSION

The overall aim of this study was to investigate the impact of age on neuroinflammation in the spinal cord of sheep, to understand the non-disease state in order to inform the development of an appropriate model of disease which is clinically translatable to humans. The results of this study showed no significant effect of age or sex on the level of astrocytic activation as shown by number of GFAP positive astrocytes. A significant difference was found however between levels of the spinal cord, where the C2 level had significantly less activated astrocytes than the C4 or T2 levels. There was also a significant decrease between the dorsal and ventral horns of the grey matter in comparison to the white matter. A significant interaction was also seen between level and region, meaning that the pattern of regional difference in astrocytes differed as a function of level of the spinal cord.

### *Age*

Our analysis showed non-significant changes in astrocyte activation with age. This suggests, contrary to research[3, 25, 26], that age may not impact astrocyte activation in an ovine model, however the relatively young age of our cohort, even in the oldest group, may have contributed to this insignificant result.

Pre-clinical rodent research showing age-dependant changes in inflammatory cell numbers, specifically microglia, in the spinal cord were significantly different only between the youngest (approximately two months) and oldest (approximately two years) age groups[44-46]. However, some studies were unable to see any significant cellular change in inflammation, and saw little change in pro-inflammatory cytokines, concluding that the aging spinal cord differs significantly from the aging brain[6]. This is an important distinction, as many of the inflamm-aging studies that include the CNS focus specifically on the brain and on microglia, and thus it is not unexpected that no significance was found in the number of astrocytes in the spinal cords of sheep that only reached middle age relative to their lifespan.

The age at which human neurophysiology changes to display clear signs of inflamm-aging is from approximately 65 years onwards, which is furthered by the increased prevalence of neurological and neurodegenerative disorders in Australians of this age group[47]. In pre-clinical models, 'aged' is a relative term associated with the lifespan of the animal being studied. The lifespan of merino sheep is approximately 12 years[48], and the oldest group of animals used for this study was 5-6 years, the animals were approximately middle aged. As such, it is not unexpected that this study did not find a significant difference in the number of activated astrocytes between age groups. Accordingly, further studies are warranted that utilise older cohorts to better investigate the impact of age.

***Sex***

Contradictory to previous studies, the current analysis showed no significant changes in astrocyte activation between the male and female groups. Indeed, rodent studies that have determined that inflamm-aging occurs in a sex-dependant manner[34, 35, 37, 49]. Nacka-Aleksić *et al,* measured microglial activation and the level of interleukin 6 (IL-6) in the plasma of young and old rats, determining that both microglial activation and IL-6 levels were significantly higher in older rats and higher in females than males regardless of age[34]. As these studies have focused on outcome measures other than astrocytes, and used different animals at larger age intervals, it is not unprecedented that this study found no statistical significance in the number of activated astrocytes between the sexes.

***Level***

This study showed a significant difference in inflammation between levels of the spinal cord, with C2 containing significantly few astrocytes compared to C4 and T2. The cervical and thoracic regions of the spinal cord are neuroanatomically distinct, with notable differences in the composition of grey and white matter, the organisation of axonal tracts, and the presence of limb-specific motor pathways[50]. Compared to the thoracic spine, the cervical spine has smaller vertebrae, increased motility, higher vascular supply and flow, and a higher grey-white matter ratio,

which results in different pathophysiological responses to stress and injury[51, 52]. The sheep spine in general is incredibly flexible, and it requires extensive stabilisation, which occurs mainly at the C2-C3 level[53]. Due to the flexibility of the sheep spine at the C2 level, the spinal cord is at increased risk of injury due to the increased range of motion and strain[53]. As a role of astrocytes in the spinal cord is to regulate the BSCB by encapsulating all blood vessels, the combination of higher vascularity and increased risk of injury in the upper cervical (C2) level, the results of this study are in direct contrast to the literature[51-53].

Nevertheless, these results raise potential issues in the use of quadrupeds as animal models for disorders of the spinal cord due to the differences in the structure of the skeletal, vascular and neurological anatomy mentioned above, which could potentially impact the results seen in levels of inflammation, due to differences in physical functionality. A potential future direction would be the comparison of the cervical neuroanatomy of the ovine model and rodent model to the human cervical spinal cord to provide an understanding of differences that exist and may need to be taken into consideration when studying the neuroinflammatory response of the cervical spinal cord.

***Region***

This study demonstrated a significant regional difference, with a greater number of astrocytes in the grey matter (dorsal and ventral horn) compared to all white matter regions. Furthermore, within the white matter, there was a significantly higher number of astrocytes in the ventral column compared to the lateral column.

The grey matter (GM) of the spinal cord contains the cell bodies of somatic, visceral, and motor nuclei, as well as containing the synapses for nuclei of the spinal roots. The large number of cell bodies in the GM requires greater astrocytic activity to regulate the BSCB, as well as the mediation of the synaptic clefts[20]. In contrast, the white matter (WM) majorly consists of myelinated axons, forming neuronal tracts. Therefore, given the higher numbers of astrocytes in the GM, and the impact of inflamm-aging being consistent activation across the cell type, the significant differences between the GM (dorsal and ventral horns) and the WM are expected.

Within the WM, the number of activated astrocytes was significantly higher in the ventral column than in the lateral column. The localisation of specific neural tracts has not been thoroughly researched in the ovine model, and as such it is possible that this difference is due to a higher density of axons in particular tracts through the spinal cord, or locations of high cellularity. This has been seen in other large animal models such as pigs, where the location and function of the corticospinal tract was more similar to that of humans than rodents[43]. As astrocytes form a link between the blood vessels and neurons of the spinal cord, they are the primary storage site for energy metabolites, and it is now understood that in times of high neuronal activity, astrocytes provide energy substrates to continue neuronal activity[54]. This suggests that there is a link between areas of high neuronal activity and a requirement for a larger number of astrocytes, which could potentially explain the regional differences in astrocyte numbers seen in this study. This is another potential future direction as the location of specific tracts may be critical for developing models of spinal cord disorders to improve clinical translation.

***Conclusion***

This study has demonstrated that level and regional differences in astrocytes exist within sheep, an important finding for modelling of future spinal cord disorders within large animals. It is important to note the limitations of this study, such as (1) the small sample size, (2) perfusion methods resulting in primarily brain perfusion only, and (3) lack of phenotypic astrocyte characterisation. These may all have impacted the results and should be considered within future studies.

To further elucidate the relationship between age and neuroinflammation within the spinal cord, evaluation of microglial activity and associated cytokines is needed. This would provide further evidence for the use of large animal models to potentially bridge the gap between experimental and clinical outcomes. Finally, the significant results of this study in relation to differences in inflammation between the anatomical spinal levels and regions provides an indication that differences in spinal structure and spinal tract location should be investigated between humans

and commonly used animal models, such as rodents, pigs and sheep, to confirm suitability of these animal models for spinal cord research. *4497 words*

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