# EVALUATION OF THE NEUROPROTECTIVE EFFECTS OF SIMVASTATIN AGAINST ALCOHOL-INDUCED DAMAGE TO THE SCIATIC NERVE AND THE SOMATOSENSORY BARRELS IN ADOLESCENT C57BL/6J MICE

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Master of Science in Medicine by Research

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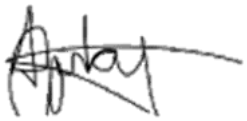
## DECLARATION

I Alice Adetokunbo Efuntayo declare that this Dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



5th day of June 2023 in Johannesburg, South Africa

## DEDICATION

To Almighty Yahweh for His unending grace

To my loving parents for their endless support

To my wonderful family

## CONFERENCE PRESENTATION ARISING FROM THIS DISSERTATION

Evaluating the neuroprotective effects of Simvastatin against Alcohol–induced peripheral neuropathy in adolescent C57BL/6J mice by **Efuntayo AA**., du Preez Robin & Olateju O.I.

*Poster presentation at the University of the Witwatersrand Faculty of Health Sciences Research Day (2022)*

## PUBLICATIONS ARISING FROM THIS DISSERTATION

There is presently no publication that has arisen from this dissertation. However, two manuscripts are currently being prepared for submission to ISI–DHET accredited journals.

I) **Efuntayo A**, Du Preez R, Ali H & Olateju OI. Simvastatin significantly prevented the onset of alcohol–induced peripheral neuropathy in the adolescent mouse.

II) **Efuntayo A**, Du Preez R & Olateju OI. Simvastatin significantly prevented barrel–specific alcohol damage in the somatosensory cortex of the adolescent mouse.

## ABSTRACT

Alcohol is a commonly used and abused drug among adolescents which has an adverse effect on the body's overall health, especially on the developing brain. It causes neurodevelopmental, neurobehavioral, neurocognitive, and social problems because alcohol exerts its neurodegenerative effects by up-regulating oxidative stress which is responsible for neuronal death. The rising prevalence of alcohol-related diseases and disabilities and the cost to the government necessitates investigation into interventions that could protect the neurons against the damaging effects of alcohol. One drug with antioxidant properties is Simvastatin, a U.S. Food and Drug Administration (FDA) approved drug for lowering blood cholesterol levels. The neuroprotective effects of Simvastatin against alcohol neurotoxicity were evaluated on the sciatic nerves and the somatosensory barrel cortices of adolescent mice. 40 four–week old C57BL/6J male and female mice were administered 20% alcohol (i.p.), 5 or 10 mg/kg Simvastatin orally followed by 20% alcohol (i.p.) or the controls (i.e. 5 mg/kg Simvastatin only or non-treated) consecutively for 28 days. The axonal density, myelin thickness and g-ratio of the sciatic nerves were assessed as well as the sizes of the Posteromedial barrel subfield (PMBSF) barrels. The results confirmed alcohol neurotoxicity on the axonal density and myelination in both sexes. At the same time, Simvastatin was effective against the onset of alcohol nerve damage. For the somatosensory barrels, alcohol did not significantly reduce the mean areas of (I) the PMBSF barrels, (II) the enclosure, or (III) the septal portion in both sexes. However, the barrel-to-barrel comparison revealed alcohol toxicity on specific barrels in specific rows and arcs of the PMBSF barrels. Both concentrations of Simvastatin were also effective against alcohol–induced damage on those specific barrels. These may explain the reasons for the sensory-motor delays that are often seen in alcoholics due to possible delays in the relaying of sensory input and the processing and interpreting of information from the somatosensory cortex. Simvastatin seems to have the ability to protect against the damaging effect of alcohol on the peripheral nerves and the somatosensory cortex and this may be beneficial in reducing the prevalence of alcohol-related diseases or disabilities, especially in adolescents that are prone to abusing alcohol.

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**NOMENCLATURE**

**ALC:** Alcohol- treated experimental group

**ALC+SIM5:** Alcohol and 5mg/kg Simvastatin treated experimental group

**ALC+SIM15:** Alcohol and 15mg/kg Simvastatin treated experimental group

**APN:** Alcohol-related peripheral neuropathy

**BAC:** Blood alcohol concentration

**NT:** Non-treatment experimental group

**PMBSF:** Posteromedial barrel subfield

**SIM:** 5mg/kg Simvastatin treated experimental group

# CHAPTER 1

## 1.1 Introduction

Alcohol abuse has adverse effects on the overall health of the body as it affects essential organs such as the heart (alcoholic cardiomyopathy), the liver (alcohol–induced cirrhosis), and the brain (diverse brain disorders and impairments) (Oscar-Berman et al., 1997; Huang et al., 2011 Fernández-Solà, 2020). Alcohol is the most used and abused drug among youths and its availability to the underaged has ignited its abuse by adolescents which has now become a serious public health concern (WHO, 2004). Adolescence is the transitional phase between childhood and adulthood (Spear, 2018) and it is a critical period during which cultural, economic, and psychosocial factors change, shape, and influence the behaviour of adolescents (Enright et al., 1987; Vadeboncoeur et al., 2022). The adolescence period is also characterised by remarkable biological developments such as pubertal–hormonal changes (Buck Louis *et al*., 2008) as well as developmental changes in the brain (Luna et al., 2010; Spear 2000, 2018; Squeglia et al., 2012) which is usually completed at about the age of 25 years old (Arain et al., 2013). During adolescence, there is an increase in risk-taking and novelty-seeking (Spear, 2000) resulting in experimenting with alcohol which often involves heavy episodic drinking (Marshall, 2014; Morojele & Ramsoomar, 2016). Alcohol abuse during adolescence has been shown to have adverse effects on the developing brain (Lees et al., 2020) resulting in neurobehavioral and neurodevelopmental problems such as learning impairment, difficulty with solving problems (Brown *et al*., 2008; Seemiller & Gould, 2020; Tetteh-Quarshie & Risher, 2023) and irrational decision-making, emotional problems and anxiety (Soyka, 2001). Morphologically, the brain shows significant shrinkage following chronic consumption of alcohol (de Bellis et al., 2000; Nagel et al., 2005).

## 1.2 Prevalence of adolescent alcoholism

On average, adolescents drink two times more than adults per occasion (NSDUH, 2007) and the prevalence of hazardous drinking habits continues to rise among adolescents (SAMSHA, 2018). Globally, 26.5% of all adolescents between the ages of 15 and 19 years are alcoholics (WHO, 2018). This age group also makes up the highest proportion of alcoholics in Europe, America, the Western Pacific, and Africa (WHO, 2018) of which most adolescents had initiated drinking between 12 and 13 years old (Jernigan, 2001; Reddy et al., 2013; Kann et al., 2018; Baiden et al., 2021). The South African Department of Social Development reported that

alcohol is also the most abused drug in South Africa (SADSD, 2010) evidenced by a high prevalence of heavy alcohol consumption among its youths (Parry *et al*., 2002; Carney *et al*., 2013; Reddy *et al*., 2013). According to the National Youth Risk Behaviour Survey (YRBS) carried out amongst high school South African learners from grade 8 to 11, about 49.2% had drunk alcohol, about 25.1% had binged on alcohol within 30 days before the survey and about 12.4% had initiated drinking before the age of 13 years (Reddy et al., 2013). Studies have shown that the earlier an individual initiate alcohol consumption, the more likely they will become alcohol dependent later in life (Grant & Dawson, 1998; Maimaris & Mccambridge, 2014). Interestingly, children exposed to prenatal alcohol, as is the case in Fetal Alcohol Spectrum Disorder (FASD), are also more likely to abuse alcohol later in life (Streissguth et al., 2004) due to gene transcriptional changes (i.e., epigenetics) that are caused by the teratogen (Shukla et al., 2008). This factor thus makes the effects of alcohol permanent, irreversible, and transferred from one generation to another (Finegersh *et al*., 2015; Rompala & Homanics, 2019).

### 1.3 General adverse effects of alcohol

Alcohol has been shown to damage essential organs such as the heart, the liver, (Huang et al., 2011; Fernández-Solà, 2020), the digestive tract (Haas et al., 2012) as well as the nervous system resulting in neurological disorders such as Wernicke disease, Korsakoff psychosis (Victor, 1992) and sensory–motor disorders (e.g., paresthesia, dysesthesia, and ataxia) (Chopra & Tiwari, 2012; Pullen & Ruiz, 2019). The effect of alcohol on the nervous system has been reported to be more severe in adolescents than in adults (Acheson et al., 1998; Markwiese et al., 1998). Alcohol abuse during the adolescent period also has adverse effects on the central nervous system (Lees et al., 2020) which may cause a downstream effect on the peripheral nervous system or vice versa (Mellion *et al*., 2011). Clinical presentations of the adverse effects of alcohol on the brain (i.e., neurological disorders such as alcohol–related psychosis) and on the peripheral nerves (i.e., sensory–motor disorders such as paresthesia) can reduce an adolescent's learning capacity due to impaired learning and memory capacity (Hanson *et al*., 2011; Jacobus & Tapert, 2013) and/or impaired fine motor skills causing a tingling feeling or burning pain felt in the hands (difficulty in holding a pen) (Chopra & Tiwari, 2012).

#### 1.3.1 Alcohol–induced peripheral neuropathy

Alcohol abuse induces distal axonopathy (Yu & Koh, 2017). Distal axonopathy (also known as dying-back neuropathy) is a type of neurotoxic neuropathy (Spencer & Schaumburg, 1978) that is characterised by a slow degeneration of the distal end of the affected axons wherein the

degenerative process would slowly progress towards the cell body of affected nerves (Moloney et al., 2014). This type of peripheral neuropathy is associated with metabolic dysfunction (diabetes) (Thomas, 1999), deficiency syndrome (malnutrition) (Dudek *et al*., 2020), or toxins (drugs or alcohol) (Griffin, 1992). Distal axonopathy induced by alcoholism is known as alcohol–related peripheral neuropathy (APN) (Yu & Koh, 2017).

APN results in sensory, motor, and autonomic dysfunctions (Mellion et al., 2011) which are characterised by sensory loss, muscle weakness, and burning sensation in the hands and feet (Hughes, 2002). It affects the lower limb more than it affects the upper limb as distal axonopathy is common in the distal nerves of the lower limb in alcoholics (Koike & Sobue, 2006). APN progresses slowly over time and often presents with the loss of small nerve fibers causing sensory–related polyneuropathy symptoms (Koike & Sobue, 2006) such as numbness, paresthesia (pins and needles), and decreased pain threshold (hyperalgesia and allodynia) (Chopra & Tiwari, 2012). APN can also be due to a direct dose–dependent alcohol neurotoxicity on the peripheral nerves or a result of a thiamine deficiency (Monforte *et al*., 1995; Koike & Sobue, 2006; Sechi & Serra, 2007).

A direct dose–dependent alcohol (and its metabolites) neurotoxicity causes up–regulation of mitochondrial free radicals, circulating cytokines, and oxidative stress which are responsible for the degeneration of axons and demyelination of sensory and motor nerves (Dina *et al*., 2000; Chopra & Tiwari, 2012). In addition, alcohol is metabolised into acetaldehyde (by alcohol dehydrogenase and cytochrome P450) (Chopra & Tiwari, 2012) which is also toxic and highly reactive in direct alcohol neurotoxicity (Chopra & Tiwari, 2012). When alcohol is oxidized by cytochrome P450 into acetaldehyde, there is an increase in reactive oxygen species which in turn, up–regulates oxidative stress (Zima, 1993; Preedy *et al*., 1999; Das & Vasudevan, 2007). Oxidative stress is one way through which alcohol exerts its neurodegenerative effects by triggering a series of sequential events that results in a significant loss of neurons (Brocardo *et al*., 2011). Alcohol also causes an imbalance between pro-oxidants and anti-oxidants which then damages biological molecules such as DNA, fats, or proteins leading to APN (McDonough, 2003; Mansouri *et al*., 2022).

In thiamine deficiency in alcoholics (McCombe & McLeod, 1984; Yu & Koh, 2017), alcohol does not only reduce the intestinal absorption of thiamine and/or its storage in the liver but it also prevents the conversion of thiamine into its active form – thiamine pyrophosphate, an active co-enzyme in the metabolism of carbohydrates and lipids (Brin, 1964; Martin et al.,

2003). Thiamine (also known as vitamin B1) is an essential micronutrient that is responsible for cellular/neuronal energy production because it serves as a co–factor for some thiamine-dependent enzymes which are involved in energy production (Lonsdale, 2006; Tylicki et al., 2018). Some thiamine–dependent enzymes are also involved in the production of neurotransmitters (Vernau *et al*., 2015) and the synthesis of anti-oxidative stress agents (Turan *et al*., 2014) such as pyridoxal phosphate (Mehta *et al*., 2008). Thiamine is also important for metabolism in the cerebrum, thus its deficiency results in polyneuropathic diseases such as Wernicke encephalopathy (dry beriberi) (Butterworth, 1989) as well as thiamine deficiency–related peripheral neuropathy which causes a progressive loss of large axons resulting in motor–related problems such as muscle cramps/spasms and weakness, muscle atrophy, muscle weakness and impaired limb movement (Chopra & Tiwari, 2012; Dudek *et al*., 2020).

In alcohol–related studies, alcoholism is associated with a reduction of axonal density (Koike et al., 2003) and demyelination of axons (Koike et al., 2001) in the sural nerve in humans. A decrease in axonal size (Mellion et al., 2013) and a reduction in axonal density in the sciatic nerves of adult rats (Bosch et al., 1979; Mellion et al., 2013; Kashkin et al., 2016) have also been reported. Interestingly, compensatory mechanisms triggered by a significant loss of axons in peripheral nerves due to alcohol neurotoxicity was observed in the sural nerve of humans diagnosed with APN (Koike *et al*., 2001) and in the sciatic nerve of adult rats administered alcohol (Nguyen et al., 2012; Mellion et al., 2013) evident by an increase in the number of regenerative axonal sprouts.

In addition, studies have also reported demyelination of axons in the sciatic nerve of adult rats that were administered alcohol thus disrupting neuron function (Nguyen *et al*., 2012; Kashkin *et al*., 2016). In other studies, no evidence of demyelination by postnatal alcohol exposure was reported in rats (Phillips, 1989) or young adult mice (Juntunen et al., 1983) following myelin thickness measurement. Another important factor used to determine the extent of peripheral neuropathies (Rodrigues *et al*., 2014) is the g–ratio which defines the degree of myelination (Rushton, 1951). This is defined as the ratio of the inner axon diameter to the outer diameter of the axon. A theoretical optimal g–ratio of 0.6 was first reported by Rushton (1951) from calculations based on the velocity of signal conduction of axons (Rushton, 1951) and subsequently, an optimal g–ratio was reported to be in a range of 0.6 to 0.75 (Berman et al., 2018). The g–ratio is an important parameter for evaluating myelinated axons because the g–ratio is physiologically optimized to ensure the maximal efficacy of a nerve (Thomas & Ochoa, 1984). Determining the g–ratio of axons in both healthy and pathologic peripheral nerves gives

an indication of the functional integrity of the nerve (Cercignani et al., 2017) which is useful for evaluating techniques and interventions for nerve repair and/or assessing the course of regeneration post-injury (Ugrenović et al., 2016).

Alcoholism is associated with a reduction of g-ratio in a study to evaluate the differences in alcoholic and thiamine-deficiency neuropathies (Koike et al., 2003). The study revealed that the calculated g–ratio of axons in the sural nerve of two groups of patients (APN with no thiamine deficiency vs APN with thiamine deficiency) was significantly reduced when compared to the control group (Koike et al., 2003). However, the calculated g–ratio of axons was found to be similar in the sciatic nerves of young adult mice (Juntunen et al., 1983) and in the optic nerves of rat pups (Phillips, 1989) that were exposed to alcohol.

Alcohol effects have also been reported in other nerves. For example, Kjellström & Conradi (1993) reported a decrease in the mean cross–sectional area of the optic nerve of adult rats, but no difference was observed in the axonal density following alcohol exposure. Also in the optic nerve, the number of axons and the size of the nerve were significantly reduced following alcohol exposure in adult rats (Igit & Colcimen, 2019) and rat pups (Phillips, 1989) Similarly, Dursun *et al*. (2013) reported a significant decrease in the population of neurons in the ganglion cell layer of the retina and the dorsolateral geniculate nucleus of mice pups following alcohol administration.

### 1.3.2 Alcohol–induced brain damage

Alcohol also has a damaging effect on the brain. It is well documented that excessive alcohol consumption over a long period leads to white matter atrophy and neurodegeneration due to the degradation of myelin structure (Harper, 2009; Yalcin et al., 2017). Demyelination is associated with a decrease in brain volume (Daviet *et al*., 2022) and loss of brain function in the affected areas (Harper & Kril, 1985; Pfefferbaum & Sullivan, 1997) leading to brain disorders e.g., cognitive impairment (Crews, 2008). In adolescent alcoholics, the brain regions most susceptible to damage are the cerebral cortex, limbic system, and cerebellum as these areas are still developing (Squeglia et al., 2009). Severe brain damage to the prefrontal cortex results in an inability to control emotions such as in alcohol–related psychosis and frontal lobe syndrome (Soyka, 2001). Severe brain damage to the limbic system [which is crucial for memory (Oscar-Berman *et al*., 1997), homeostasis, and expressing emotional feelings (Oscar-Berman & Marinković, 2007)] results in functional neurological disorders such as amnesia and abnormal emotion (Evert & Oscar-Berman, 1995; Oscar-Berman *et al*., 1997; Kornreich *et al*., 2002).

Learning and memory are also impaired due to damage to the hippocampus in adolescent alcoholics (de Bellis *et al*., 2000; Nagel *et al*., 2005; Mellion *et al*., 2011; Anand & Dhikav, 2012). In addition, damage to the cerebellum by alcohol hinders motor coordination e.g., alcohol–related cerebellar ataxia (Sullivan et al., 1995; Shanmugarajah et al., 2016). Another brain area often affected by alcohol is the somatosensory cortex (Kril et al., 1997; Mattson et al., 2001 Degiorgis et al., 2022).

#### 1.3.2.1. Somatosensory cortex

The somatosensory system is a part of the nervous system that processes information (tactile and sensory stimuli) from several modalities of somatic sensation. This information is specifically processed in the primary somatosensory cortex (S1) wherein tactile representation (called homunculus) of the body is located (Borich *et al*., 2015; Harding-Forrester & Feldman, 2018). The S1 processes sensory information and then sends the processed information via short association fibres to the motor cortex to initiate action/movement (Catani et al., 2012; Borich et al., 2015). Therefore, improper or altered processing of sensory inputs by the S1 may affect the normal functioning of the primary motor cortex (M1) (Borich *et al*., 2015). In the event of defective processing and translation of sensory information from S1 to M1 before and/or during movement, the resulting motor behaviour may be abnormal and/or imprecise (Borich *et al*., 2015). This emphasizes the important association/connection between sensory and motor functions (Edwards et al., 2019) and more broadly the association between the somatosensory cortex and the peripheral nerves (which are also affected by excessive alcohol consumption causing APN) (Ammendola et al., 2001; Caspers et al., 2011; Mellion et al., 2011). This connection has also been implicated in several neurologic motor dysfunctions due to impaired sensory processing (e.g. stroke, Parkinson's disease, dystonia, or ataxia (Kalant *et al*., 1975; Elbert *et al*., 1998; Lewis & Byblow, 2002; Konczak & Abbruzzese, 2013; Zhang *et al*., 2021) as damage to the S1 area results in deficits in psychomotor abilities such as in fine motor movements where intricate finger movements is impaired hindering the ability to identify objects by feeling (e.g. shape, size, and texture) (Freund, 2003).

#### 1.3.2.2 Morphology of the somatosensory cortex

In humans, the somatosensory cortex is located on the postcentral gyrus of the parietal lobe where the ‘somatographic’ representation/map of the contralateral side of the human body (the homunculus) is located (Muret *et al*., 2022; Nguyen & Duong, 2022). The homunculus is organised in an inverted manner such that the ‘somatographic’ representation of the toes is at the superior part of the gyrus while the mouth is represented at the inferior part of the gyrus

(Nguyen & Duong, 2022). The size of the S1 area dedicated to the representation of a body region(s) is proportional to the density of sensory receptors located in that region and the number of somatosensory inputs from that area (Catani, 2017)which is why the hands, lips, and mouth have the largest representation than other parts of the body on the gyrus (Catani, 2017). In rodents, the somatosensory cortex is similar except that the somatosensory cortex in rodents consists of cell aggregates called somatosensory barrels (Erzurumlu & Gaspar, 2020). The somatosensory barrels also receive (via sensory nerves) and process sensory information from the contralateral side of the body of rodents e.g., vibrissae (whiskers) and paws (Woolsey, 2016; Adibi, 2019). The connections (between sensory input and somatosensory barrels), neural organization, and topography of barrels are well developed in rodents which thus makes the somatosensory system in rodents a suitable means of evaluating brain functions (Erzurumlu & Gaspar, 2020), plasticity (Erzurumlu, 2003), learning (Erzurumlu & Gaspar, 2020), sensory capability (Jones et al., 2019) and cortical connections (Erzurumlu & Gaspar, 2020).

The barrel cortex in rodents was first discovered in layer IV of the cortex by Lorente de Nó (1922) and later organized into 'barrels' by Woolsey & Van der Loos (1970) who described them as dense elliptic-shaped neuronal aggregates surrounded by inter-barrel septa consisting of corticocortical fibers (Feldmeyer *et al*., 1999; Kim & Ebner, 1999; Petersen & Diamond, 2000; Chakrabarti & Alloway, 2006). The barrels are organized into barrel subfields (Woolsey & van der Loos, 1970; Welker, 1976) and larger subfields generally represent areas of the body that are more active and require more cortical area for processing (Chappell *et al*., 2007). Six major barrel subfields have been identified in rodents (Seelke et al., 2012) but the largest being the Posteromedial Barrel Subfield (PMBSF), which represents the facial vibrissae on the head of the rodent, is the focus of this study (Welker & Woolsey, 1974; Chappell *et al*., 2007).

The PMBSF barrels process tactile inputs from the contralateral long whiskers (macro-vibrissae) on the snout of rodents (Welker & Woolsey, 1974; Brecht *et al*., 1997; Oladehin *et al*., 2007). Each barrel receives and processes sensory input from 1–2 vibrissae (Woolsey & van der Loos, 1970; Powrozek & Zhou, 2005). The PMBSF barrels are organized into 5 rostro–caudal rows (A–E). The first two rows (A–B) are made up of 4 barrels each while the remaining 3 rows (C–E) are made up of 5 barrels each (Bressler, 1995; Chappell et al., 2007) as well as four straddler barrels (i.e., barrels α, β, γ, and δ) located anterior to the first 4 rows (A–D) of the PMBSF barrels (Chappell et al., 2007). These barrels are easily identified using specialized staining (Finnerty *et al*., 1999; Petersen & Sakmann, 2000).

#### 1.3.2.3 Alcohol–induced somatosensory damage

A reduction in the volume of grey matter in the parietal lobe has been reported in humans that are alcoholics (Fein et al., 2009) as well as an impairment of sensorimotor function caused by a reduction in sensory processing (Jirikowic et al., 2008). This demonstrates the extent of neurologic damage to the S1 causing motor dysfunction in humans that are alcoholics (Spindler et al., 2021). In animal studies, prenatal alcohol exposure significantly reduced the PMBSF barrel size and significantly reduced the average individual barrel size of pups, and adolescents (Margret *et al*., 2005; Powrozek & Zhou, 2005; Chappell *et al*., 2007). A similar effect was reported in rat pups exposed to postnatal alcohol (Oladehin *et al*., 2007). The reduction in barrel size was specific to a region of the PMBSF barrels as the anterior barrels are more vulnerable than the posterior barrels which indicates regional vulnerability to alcohol (Margret et al., 2005; Chappell et al., 2007). However, Powrozeka & Zhou, (2005) found that the posterior barrels are more negatively affected by alcohol than the anterior barrels with some posterior barrels not developed in rows A and B as well as in the straddler barrels. Similarly, Olateju *et al*. (2019) also reported a significant reduction in the size of barrels in specific rows following prenatal alcohol exposure despite reporting no significant difference in the average total area of the PMBSF and the average individual barrel area of mice. The septal area which contains corticocortical fibres is also vulnerable to alcohol following prenatal exposure (Margret *et al*., 2005; Powrozeka & Zhou, 2005; Margret *et al*., 2006) and postnatal exposure (Oladehin *et al*., 2007) but no significant difference was reported following a prenatal exposure on adult mice by Olateju *et al*. (2019) and on adolescent or adult mice by Chappell *et al*. (2007). There was similarly no disruption to the barrel organization or pattern (Margret *et al*., 2005; Margret *et al*., 2006; Oladehin *et al*., 2007; Chappell *et al*., 2007; Olateju *et al*., 2019).

Studies on the alcohol effect on the somatosensory barrels mostly focussed on either prenatal (Margret *et al*., 2005; Margret *et al*., 2005b; Powrozeka & Zhou, 2005; Margret *et al*., 2006; Chappell *et al*., 2007; Olateju *et al*., 2019) or postnatal exposures to alcohol (Oladehin *et al*., 2007) and the alcohol effects evaluated at different developmental stages e.g., pup (Powrozeka & Zhou, 2005; Margret *et al*., 2005; Margret *et al*., 2005b; Margret *et al*., 2006; Oladehin *et al*., 2007; Olateju *et al*., 2019), adolescent (Chappell et al., 2007) or adult (Chappell et al., 2007). This creates a knowledge gap on the effect of alcohol on the development of the somatosensory system, especially in adolescents whose brains are still developing and who often experiment with alcohol during this period of brain growth. To re-emphasize, alcoholics often develop sensorimotor problems due to the vulnerability of the somatosensory cortex, primary motor, and peripheral nerves to alcohol (Erzurumlu, 2003; Li & Crair, 2011; Jones *et al*., 2019;

Erzurumlu & Gaspar, 2020). Due to the diverse vulnerability of the somatosensory cortex, alcohol–related studies often involve investigating the morphology of the somatosensory cortex (Margret et al., 2005; Margret et al., 2005b; Margret et al., 2006; Chappell et al., 2007; Oladehin et al., 2007; Olateju et al., 2019)

#### 1.3.2.4 Interventions against alcohol–induced damage

Alcohol exerts its neurodegeneration effects through the upregulation of oxidative stress (Haorah et al., 2008) which then initiates a sequence of events that cause significant damage to neurons of the central nervous system and peripheral nerves (i.e., neurodegeneration) (Pervin et al., 2021). Alcohol is also implicated in causing genomic instability in neurons (neurodegeneration) by affecting some metabolic pathways such as the one–carbon metabolic pathway (OCM) which is responsible for the biosynthesis of DNA precursors (for DNA repair) and methyl groups (for methylation) (Fowler et al., 2012; Kruman et al., 2012). OCM dysfunction is associated with alcohol exposure and is considered a significant factor in alcohol–induced neuropathy(Fowler et al., 2012).

Acetylaldeyde is another metabolite of alcohol that is highly reactive and toxic to neurons and nerves (Koike & Sobue, 2006). It oxidizes biological molecules such as lipids (through lipid peroxidation), proteins, and DNA (Brooks, 1997; Ramachandran *et al*., 2001; Xi *et al*., 2004; Brooks & Theruvathu, 2005; Koike & Sobue, 2006). Lipid peroxidation generates lipid oxidative byproducts such as Malondialdehyde (MDA) which is considered genotoxic (Marnett, 1999) as it destroys DNA nucleotides and form DNA adducts such as pyrimido-[1,2-α]purin-10(3H)-one deoxyribose which causes sequence-dependent frameshift mutations that are associated with cancer development (Marnett, 1999). In addition, MDA was reported to be abundant in the sciatic nerves of rats chronically exposed to alcohol (Chopra & Tiwari, 2012). There have been clinical and experimental attempts to mask, prevent or reverse the neurotoxic effects of alcohol. One such way is by targeting pathways or means through which alcohol exerts its damaging effects. For example, the neuroprotective effects of anti-oxidants found in fermented *Aspalathus linearis* (Rooibos tea) against alcohol–induced oxidative stress was found to mask alcohol effects (i.e. increased viability and proliferation) on endothelial cells but had no effect on the transcription of claudin-5 (a tight junction protein of the blood–brain barrier) (Greene et al., 2019) and paracellular permeability (Hu et al., 2013). Similarly, dietary soy was found to be beneficial in preventing the degenerative effects of alcohol on the behaviour functions of the brain as well as on insulin-dependent metabolic pathways in the brain, i.e., the insulin/insulin-like growth factor type 1 (IGF-1)-Akt pathway, in the brain of

adolescent male and female Long Evans rats (Tong et al., 2022). The IGF-1-Akt signaling pathway is affected by alcohol and its impairment is associated with alcohol–induced neurodegradation, a functional decline of the adolescent brain (Ewenczyk *et al*., 2012; Tong *et al*., 2014; Tong *et al*., 2022) and alcohol–related myopathy (Nguyen et al., 2012). Tian *et al*. (2016) also reported that Sodium vitamin C transporter 2 (SVCT2) [which is imperative for the transportation of L-ascorbic acid, Vitamin C (Gess et al., 2010)] has a beneficial neuroprotective effect against alcohol–induced oxidative stress through JNK/p38 MAPKs, NF-κB signaling and miR-125a-5p pathways in adult rats following chronic alcohol exposure. Also, vitamin B complexes (vitamin B1, B2, B6 and B12, and B9) in a clinical trial were found to improve the symptoms of APN (Peters et al., 2006).

A class of drugs that is beginning to gain popularity amongst neuroscientists is Statins e.g., Simvastatin due to its anti–oxidative and neuroprotective effects against neurodegeneration (van der Most et al., 2009; Wang et al., 2011; Chataway et al., 2014). Statins is a readily available and an FDA–approved (Food and Drug Administration) drug for reducing blood cholesterol levels by actively inhibiting the rate–limiting step in the mevalonate (MVA) pathway which is responsible for the biosynthesis of cholesterol (Silva *et al*., 2013) however they generate other active ends–products (e.g. Coenzyme Q in the mitochondrial respiratory chain, farnesyl and geranylgeranyl moieties for protein post-translational modifications, isopentenyl tRNAs for RNA transcription and dolichol for protein N-glycosylation) with several physiological functions such as neuroprotection (Segatto et al., 2014; Fracassi et al., 2019). Coenzyme Q exerts its neuroprotective effects through various cellular and systemic mechanisms that are not yet fully understood (Silva et al., 2013) however the capacity of Statin to inhibit neurotoxicity against N-methyl-D-aspartate–induced cell death (van der Most *et al*., 2009) and prevention of neurodegeneration in mouse models of Alzheimer disease (Wang *et al.*, 2011), Ischemic stroke (Montecucco *et al*., 2011), Parkinson's disease (Wang *et al*., 2011), Huntington's disease (Schultz *et al*., 2019) and Multiple sclerosis disease (Chataway *et al*., 2014) have been reported.

In addition, the ability of Statins to inhibit neurotoxicity against alcohol–induced neurodegeneration has also been reported (Nasef *et al*., 2021). It was found that Simvastatin treatment before chronic alcohol administration in female Albino Swiss mice suppressed neurodegeneration and resulted in a partial improvement of brain function (Nasef *et al*., 2021). In another study, Simvastatin was found to improve the morphology and recovery of function of the sciatic nerve of adult rats following the introduction of a crush lesion in the nerve (Xavier

*et al*., 2012. Interestingly, there is a lack of information in the literature on the neuroprotective effects of Simvastatin against alcohol–induced peripheral neuropathy and somatosensory damage in adolescent mice.

## 1.1 **Rationale of study**

With the rising prevalence of adolescent alcoholism in society and the social miss-ills associated with this problem, it is imperative to investigate interventions that could mask the damaging effects of alcohol misuse, especially where other interventions such as enlightenment campaigns and stricter access to alcohol by underage by the South African government have proven not/less effective or adequate in preventing misuse. And the cost to the government in providing health care, rehabilitation, special education, crime, and crime control associated with this problem cannot be overlooked. Considering the cost to the government and the impacts on individuals and communities, it is thus imperative to investigate readily available and cost-effective interventions (such as Simvastatin) that could mask the damaging effects of alcohol misuse. In the present study, the capability of Simvastatin to prevent the onset of alcohol–induced neuropathy and somatosensory damage in mice exposed to prolonged alcohol administration was explored.

### 1.4.1 Study aim

This study aimed at investigating whether Simvastatin could mask the damaging effect of alcohol neurotoxicity on the peripheral nerves and the somatosensory barrel cortices of adolescent mice that were exposed to chronic alcohol.

### 1.4.2 Study objectives

The specific objectives of this study are:

(I) To investigate the neuroprotective effect of treatment with Simvastatin against alcohol–induced damage on the sciatic nerve of adolescent mice that were exposed to chronic alcohol using osmium tetroxide staining.

(II) To investigate the neuroprotective effect of treatment with Simvastatin against alcohol–induced brain damage on the somatosensory barrel cortices of adolescent mice that were exposed to chronic alcohol using cytochrome oxidase reactivity staining.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1. Experimental animal study

This study was approved (Ethics Clearance No: 2019/11/63/C) by the Animal Research Ethics Committee (AREC) of the University of the Witwatersrand, Johannesburg, South Africa which parallels those of the National Institutes of Health (NIH) for the care and use of animals in scientific experimentation. Three–week old C57BL/6J mice (20 male and 20 female) were used for the study. The mice were supplied by the National Health Laboratory Service (NHLS), Johannesburg and they were housed in the animal facility of the Witwatersrand Research Animal Facility (WRAF) of the Faculty of Health Sciences, University of the Witwatersrand. This strain of mice is commonly used in alcohol–related studies and in studies to mimic fetal alcohol spectrum disorders (Olateju et al., 2017, 2018, 2019) as well as in mouse models of addiction (Alcaraz-Iborra et al., 2017; Chen et al., 2007; Depoy et al., 2016; Mulligan et al., 2011; Uhl et al., 1996). Both sexes were used in this study as studies have shown that males and females respond differently to drugs under different disease conditions (Anderson, 2008; Soldin & Mattison, 2009; Ceylan-Isik *et al*., 2010).

## 2.2. Experimental design

The animals were randomly allocated into five experimental groups: Non–treatment (NT), Alcohol (ALC), 5 mg Simvastatin (SIM), Alcohol + 5mg Simvastatin (ALC+SIM5), and Alcohol + 15mg Simvastatin (ALC+SIM15). The mice were then allowed to acclimatize for one week, following which alcohol, Simvastatin, or appropriate controls were administered to the mice (at 4 weeks old) for a period of 28 days according to Table 1. Alcohol was administered intraperitoneally while Simvastatin was administered via oral gavage. Both procedures are performed routinely with utmost care in animal studies and these procedures were performed by the trained staff of WRAF to reduce introducing stress in the animal. Mice of the same sex and belonging to the same experimental group were housed together in a group of six mice per cage (cage dimensions: 200 × 200 × 300 mm) and kept under a reversed 12–hour day/ 12–hour dark cycle (with the light switched off from 06:00 – 18:00). For this study, the period of adolescent in the mice was within the adolescent period (i.e. 3 – 8 weeks) reported by Hefner and Holmes (2007), Lespine and Tirelli (2017).

### 2.2.1. Preparation and administration of Simvastatin

Simvastatin (Cat no: 1612700, Merck, South Africa) is a slightly yellowish substance that is insoluble in water, but it is soluble in solvents such as chloroform, methanol, and alcohol (Sopyan et al., 2018). To ensure the dissolution of Simvastatin, the drug was prepared according to McKay et al. (2004). A stock solution of Simvastatin was prepared by dissolving 80 mg of powdered Simvastatin in a solution containing 1 ml 0.1 M NaOH (1 ml) (acting as an emulsifier) and 1 ml absolute alcohol (1 ml) under a gently stirring for 1 hr at room temperature until the Simvastatin was completely dissolved. The stock solution was prepared every fifth day, they were aliquoted and stored at – 4 °C until use. Serial dilution of the stock solution was then used to prepare the high concentration (i.e., 4 mg/ml in distilled water) and the low concentration (i.e., 1 mg/ml in distilled water) of Simvastatin from which the final dosages (i.e., 15 mg/Kg/per day and 5 mg/Kg/per day respectively) were determined. These solutions were prepared daily and filtered–sterilized using a 0.20–µm sterile filter under a sterile condition. Any unused solution per day was discarded.

### 2.2.2. Preparation and administration of alcohol

Using a serial dilution, absolute alcohol (96%) (Sigma–Aldrich, South Africa; Cat no: SAAR2233510LP) was diluted in saline (0.9% NaCl) into 20% alcohol solution that was then administered intraperitoneally to the mice at a dose of 2.5 g/kg (Maldonado-Devincci et al., 2010). A saline solution (an isotonic solution) was used instead of distilled water (a hypotonic solution) (Turner et al., 2011) as it would mitigate the risk of the experimental animals developing abdominal oedema. The alcohol solution was also prepared daily, and the solution was filtered–sterilized using a 0.20–µm sterile filter under a sterile condition. Alcohol was administered to the ALC group intraperitoneally and also within 30 min after administering Simvastatin to the ALC+SIM5 or ALC+SIM15 experimental group. The injection site was alternated daily to reduce inducing stress or discomfort in the animal. To control for stress that may arise from handling or the administration of drugs in the mice, the mice in the NT group were not administered alcohol or Simvastatin and they were not handled throughout the experimental period (28 days) except for daily weighing or periodical cleaning of the cages which were performed by WRAF trained personnel. Alcohol was also not administered to the SIM group as it serves as a control for the effect of Simvastatin. Food and water were provided *ad libitum* to the mice, except in the NT and SIM groups, where it was withheld for 2 hrs post-intraperitoneal injection to partially control for the reduction in feeding during the period of peak alcohol intoxication in the alcohol-treated mice (Haycock & Ramsay, 2009).

Table 1: Table indicating the different experimental groups

| Groups | Experimental groups | Abbreviations of groups | No. of mice per experimental group (Female) | | Dose of administered substances | Duration of administration (days) |
|---|---|---|---|---|---|---|
| | | | Female | Male | | |
| 1 | No treatment | NT | 4 | 4 | No alcohol, no Simvastatin<br>To control for the procedures and treatments. | |
| 2 | Simvastatin (5 mg/Kg) | SIM | 4 | 4 | A daily oral dose of 5 mg/Kg (in distilled water). To obtain a baseline effect of Simvastatin | 28 |
| 3 | Alcohol | ALC | 4 | 4 | A daily intraperitoneal dose of 2.5 g/Kg alcohol (in saline) | 28 |
| 4 | Alcohol + 5 mg/Kg SIM | ALC+SIM5 | 4 | 4 | A daily oral dose of 5 mg/Kg SIM (in distilled water) followed by 2.5 g/Kg alcohol (in saline) administered intraperitoneally | 28 |
| 5 | Alcohol + 15 mg/Kg SIM | ALC+SIM15 | 4 | 4 | A daily oral dose of 15 mg/Kg SIM (in distilled water) followed by 2.5 g/Kg alcohol (in saline) administered intraperitoneally | 28 |
| | **TOTAL** | | **20** | **20** | | |

### 2.3. Blood alcohol concentration (BAC) assay

On the last day of administration of alcohol or Simvastatin, 50 µl of saphenous blood was collected through saphenous vein bleeding on the left hind legs of all the mice in all the experimental groups except in the NT group to determine the level of blood alcohol concentration (BAC) in each mouse (Bielawski & Abel, 1997). Blood was collected from the SIM group to equilibrate the experimental groups, but BAC analysis was not performed on the blood from this group because they were not administered alcohol. Blood was collected, within 30 mins after the administration of alcohol, in heparinized capillary tubes and then stored at –80 ºC before further processing. Before the determination of BAC, the blood in the capillary tubes was stored at 4 ºC overnight before being centrifuged with Vivaspin500© 100 µm membrane tubes (Biotech, South Africa) at 5000 rpm for 15 minutes to separate the serum. The serum was extracted and then the BAC was determined using an EnzyChrom™ Ethanol Assay Kit (BioVision, South Africa) according to the manufacturer's recommendations.

### 2.4. Animal perfusion, brain, and sciatic nerve removal

At the end of the experimental period, the mice were euthanized using Euthanaze (sodium pentobarbital, 80 mg/Kg, i.p.). Thereafter, the mice were transcardially perfused with 4% paraformaldehyde (in 0.1 M phosphate buffer, PB) (PFA), and then the mice were immediately decapitated. The brain was then quickly removed from the skull, weighed, and post-fixed for 24 hr in 4% PFA in 0.1 M PB before further processing.

For the removal of the sciatic nerve, the carcasses were placed in a supine position on a dissecting board. The skin on the left hind limb was reflected to expose the muscles of the hind limb. The thigh muscles were then split longitudinally to expose the whole length of the sciatic nerve. The nerve was gently lifted using a forceps and the section of the nerve overlying the hip joint and at its bifurcation at the popliteal fossa was carefully cut and immediately fixed in 10% buffered formalin (in 0.1M PB) and stored at 4 ºC overnight before further processing.

### 2.5. Osmium tetroxide staining of the sciatic nerve

The PFA–fixed sciatic nerve was rinsed three times in 0.1 M PB for 5 minutes each. Thereafter, the nerve was immersed in 1% Osmium tetroxide (in 0.1 M PB) for 4 hr at 4 ºC. Following post-osmification, the nerves were processed using the Thermo Shandon Citadel™ 2000 automatic tissue processor (Thermo Scientific, USA) with the following schedules: dehydration in increasing alcohol series 30, 50, 70, 90, and 100% for 30 min each; clearing in chloroform for 1.5 hr and wax infiltration for 2 hr. The nerve was then immediately embedded in paraffin

wax with the nerve in an upright position and with the distal end of the nerve at the sectioning end of the mounting block. The embedded nerve was then sectioned at 5 µm using a Leica 2035 Biocut Rotary microtome (Leica Instruments, Germany). Four serial sections were floated on a water bath at 45 ºC and collected on 0.5%–gelatine coated slides and then air dried for 24 hr before the sections were cleared in xylene and cover-slipped using an Entellan mounting medium.

### 2.5.1. Morphometry of sciatic nerve

The following parameters: outer (myelin) diameter and inner (axonal) diameter, myelin thickness, g–ratio, and axonal density were assessed. For the determination of axonal density, a random field of the nerve section was photomicrographed with a camera attached to an Axioscope microscope at times 40 objective lens. The stage was moved longitudinally to prevent capturing overlapping areas of the nerve (i.e., the field of view). The captured image was fed into an ImageJ 1.47v software (NIH, USA) where a grid (102400 µm2) was overlayed onto the image to enhance the counting of axons. The number of axons per grid was recorded. The axonal density (%) was then calculated by dividing the number of axons counted by the area of the counting grid. Only axons with well-defined boundaries and identifiable myelin sheaths (unmyelinated axons were excluded from the count) that were lying in each grid or with 80% of their axonal body overlying the border of the counting grid were counted to mitigate bias and errors in the count.

For the determination of the outer and the inner diameters of the sciatic nerve, photomicrographs of axons were taken at times 100 objective lens using a digital camera attached to a Zeiss Axioscope microscope. The stage was also moved at regular intervals to avoid capturing overlapping fields. The scale bar was inscribed onto each captured image for the setting of the measuring scale of ImageJ software. Each image was fed into ImageJ software where the straight–hand tool of the software was used to measure the inner and the outer diameters of each axon by drawing a straight transverse line through the centre of the axon connecting the two ends of an axon. Each measurement was repeated and then the average of the measurements was taken as the measured diameter of the axon.

The myelin thickness was determined from the measurements of the outer and inner diameters by subtracting the inner diameter from the outer diameter. The obtained values were normalized by dividing the myelin thickness of an axon by the corresponding area of the nerve. Also, for the g–ratio, the measurements of the outer and inner diameters were used to calculate this ratio by dividing the measured inner diameter by the outer diameter. The g–ratio defines the degree

of myelination (Rushton, 1951) and it also indicates the functionality of a myelinated nerve (Cercignani et al., 2017). According to Hernendez-Reynonso *et al*. (2021), axons with a g–ratio below 0.4 are considered damaged axons. These axons are degenerating axons with an unusually thick myelin sheath (la Marca et al., 2011; Xie et al., 2014), while axons with a g–ratio higher than 0.7 are either considered regenerating axons/sprouts with a thin myelin sheath (Fancy et al., 2009) or degenerating axons with very thin myelin sheaths (la Marca et al., 2011; Xie et al., 2014). Based on these, the axons evaluated in this study were categorized into Group I: axons with a g–ratio ≤ 0.6 (a thick myelin sheath); Group II: axons with an optimal g–ratio within the range of 0.6 – 0.7 (a myelin sheath of ideal thickness); and Group III: axons with a g–ratio ≥ 0.7 (a thin myelin sheath) (Ugrenović *et al*., 2016).

### 2.5.2. Statistical analyses for the sciatic nerve

A descriptive statistic was performed to illustrate the mean (± standard deviations) of the measurements (i.e., average axonal density and average myelin thickness). A Shapiro-Wilk test was also performed for normality, thereafter, an analysis of variance (ANOVA) or a Kruskal – Wallis test was performed to evaluate the differences in each measurement across the experimental groups (NT, SIM, ALC, ALC+SIM5, and ALC+SIM15). Where an ANOVA or a Kruskal – Wallis test was significant, a post hoc analysis using a Tukey's pairwise or a Dunn's test was performed to determine where a significant difference lies. All statistical analyses were performed using a PAST 4.03 statistical program (Version 4). A significance level of 5% was used to indicate significant differences for all the statistical analyses.

## 2.6. Cytochrome oxidase reactivity staining for the somatosensory barrels

Forty (40) left cerebral hemispheres of mice from all the experimental groups were processed for cytochrome oxidase (CO) staining according to Seelke *et al*. (2012) and Olateju *et al*. (2019). The cerebellum, thalamus, and brainstem were carefully removed from each hemisphere. Each hemisphere was then carefully cut along the acute angles of the curvature of the cortex before being manually flattened gently between two glass slides to a thickness of approximately 1 mm. The flattened hemisphere was immediately cryoprotected in 30% sucrose (in 0.1 M PB) at 4 °C until equilibrium was reached after approximately 72 hr. The cryoprotected–flattened cerebri were then frozen in an OCT medium (CellPath Services Pty Ltd, UF1000) and then sectioned in the plane of the pial surface at a thickness of 120 μm using a cryostat (Thermo Scientific, HM525NX). The serial sections were collected and then stored in 0.1 M PB at 4 ºC overnight before staining for cytochrome oxidase (CO) reactivity according

to Wong-Riley (1979), Tootell and Silverman (1985), Seelke *et al*. (2012) and Olateju *et al*. 2019.

For the CO reactivity staining, the sections were placed in a 1% solution of cobalt chloride (in 10% sucrose) for 20 mins and then rinsed three times in 10% sucrose in 0.1 M PB for 10 mins each. Thereafter, the sections were incubated in a CO solution [in a 200 ml 0.1 M PB mixture: 200 mg cytochrome oxidase (Sigma, C2506), 50 mg diaminobenzidine, 40 g sucrose, and a visible amount of catalase (Sigma C-10)] at 37 °C for 3.25 hr. Thereafter, the sections were rinsed three times in 0.1 M PB for 10 mins each and then mounted on a 2% gelatine–coated glass slide, allowed to dry for 3 days, dehydrated in a graded series of alcohol, cleared in xylene, and then coverslipped with an Entellan mounting medium.

### 2.6.1. Determination of the size of the somatosensory barrel

Photomicrographs of serial sections of the PMBSF barrels of each mouse were taken at a 5 times objective lens using a Nikon SMZ1500 dissecting microscope with an attached Nikon Digital Sight camera. Similar to other reports on somatosensory barrel organisation (Chappell *et al*., 2007; Olateju *et al*., 2019), the PMBSF barrels were identified by 4 straddler barrels (α-δ), 4 barrels in rows A and B, as well as 5 barrels in rows C, D, and E. The PMSBF barrels were mostly visible in about 1 – 2 sections from the same mouse hemisphere. The settings on the microscope were kept constant for the imaging of all the barrels in all the mice. Each captured image was inscribed with a scale bar for barrel morphometry. Where the PMBSF barrel was captured in two serial sections, the photomicrographs were aligned using the white circles that appeared on the images. These white circles are the cerebral blood vessels (Olateju *et al*., 2019). The positions of blood vessels in the photomicrographs were used to align the 27–PMBSF barrels by carefully overlaying the photomicrographs using Photoshop software (Adobe Photoshop, C6S Version 13.0.) in order to produce a single image that is representative of all the 27–PMBSF barrels for each hemisphere (Oladehin *et al*., 2007; Olateju *et al*., 2019). No alteration to the images was done during this process other than brightness and contrast adjustments for clarity of the somatosensory barrel boundaries. All the captured images were saved in a JPEG image file at 300 dpi.

The morphometries of the PMBSF barrels were performed using ImageJ software. The following parameters were measured on each image: the area of the PMBSF enclosure and the area of the individual PMBSF barrel. Then the total septal area of the PMBSF barrels was calculated by subtracting the total area of the 27–PMBSF barrels from the area of the PMBSF enclosure. The measured parameters are illustrated in Fig. 1. In addition, barrel–to–barrel

comparisons were performed across all experimental groups to reveal any size differences in the individual barrels.

### 2.6.2. Statistical analyses for the somatosensory barrels

Descriptive statistics were performed to illustrate the median and mean (± standard deviations) of the measurements (i.e., the average area of individual PMBSF barrel, the average total area of the 27–PMBSF barrels, the average total area of PMBSF enclosure and the average total septal area of the PMBSF). A Shapiro-Wilk test was also performed for normality, thereafter, an ANOVA or a Kruskal – Wallis test was performed to evaluate differences in each measured parameter across the different experimental groups. In addition, a barrel–to–barrel comparison across the experimental groups was performed. Where an ANOVA or a Kruskal – Wallis test was significant, a post hoc analysis using Tukey's pairwise test or Dunn's test was performed to determine where a significant difference lies. All statistical analyses were performed using a PAST 4.03 statistical program (Version 4). A significance level of 5% was used to indicate significant differences for all the statistical analyses.

# CHAPTER 3

# RESULTS

## 3.1. General observations on the body and brain of mice

All the mice that were administered alcohol or Simvastatin did not show any phenotypic evidence of adverse effects (such as craniofacial abnormalities or a significant reduction in body mass) of the administered substances. At the time of the sacrifice, the average body or brain mass for the different experimental groups is shown in Table 1. No statistical difference was observed across the experimental groups in terms of body or brain mass when mice of the same sex were compared.

Table 2: An illustration of the average body and brain masses of the mice in each experimental group

| Group | Sex | Number of animals (n) | Average body mass (g) | Standard deviation (g) | Average brain mass (g) | Standard deviation (g) |
|---|---|---|---|---|---|---|
| NT | Female | 3 | 15.17 | 0.29 | 0.42 | 0.02 |
| | Male | 3 | 18.33 | 0.29 | 0.41 | 0.03 |
| SIM | Female | 3 | 14.33 | 1.26 | 0.40 | 0.01 |
| | Male | 4 | 17.75 | 1.19 | 0.41 | 0.03 |
| ALC | Female | 3 | 13.67 | 2.36 | 0.42 | 0.02 |
| | Male | 4 | 17.63 | 1.80 | 0.41 | 0.02 |
| ALC+SIM5 | Female | 4 | 15.00 | 2.42 | 0.36 | 0.07 |
| | Male | 3 | 17.00 | 1.50 | 0.37 | 0.05 |
| ALC+SIM15 | Female | 3 | 13.00 | 0.50 | 0.41 | 0.02 |
| | Male | 4 | 18.00 | 1.00 | 0.42 | 0.01 |

### 3.2. Blood Alcohol Concentration (BAC)

For illustrative purposes and to confirm that each mouse received alcohol, the BAC level was determined on the last day of alcohol administration. BAC level was only assayed for mice in the experimental groups that received alcohol intraperitoneally (i.e., ALC, ALC+SIM5, or ALC+SIM15). The average BAC level for each experimental group is shown in Table 2. The highest BAC level was reported in the ALC+SIM15 group in the female (210.63 ± 55.95 mg/dl) and the ALC+SIM5 group in the male (315.46 ± 10.02 mg/dl) while the lowest BAC was recorded in the ALC group for both the female (93.12 ± 22.31 mg/dl) and the male (191.27 ± 42.27 mg/dl) mice. The BAC in each mouse was within the range reported for other studies e.g., Chappell et al. (2007), Margret et al. (2005), and Olateju et al. (2017; 2019).

Table 3: An illustration of the average blood alcohol concentration (BAC) for each experimental group that was administered alcohol intraperitoneally

| Group | Sex | Average BAC level (mg/dl) | Standard Deviation (mg/dl) |
|---|---|---|---|
| ALC | Female | 93.12 | 22.31 |
| | Male | 191.27 | 42.27 |
| ALC+SIM5 | Female | 145.70 | 26.03 |
| | Male | 315.46 | 10.02 |
| ALC+SIM15 | Female | 210.63 | 55.95 |
| | Male | 204.62 | 5.78 |

### 3.1. Morphometry of sciatic nerve

#### 3.1.1. General observation of the sciatic nerve

The myelin sheath of the sciatic nerve appeared dark following an osmium tetroxide staining. The axons which were identifiable by their dark–stained myelin sheaths were enclosed in a lightly– stained epineurium. These axons were mostly round to oval with intact myelin sheath which was supported by a lightly stained endoneurium but lacked a distinguishable perineurium. The myelinated axons varied in size in each nerve and these observations were obvious and similar in all the nerves across the different experimental groups. A representative photomicrograph of the sciatic nerve and the myelinated axons are shown in Figure 1. The myelinated axons across the experimental groups were without visible signs of neuropathy and were thus considered typical of the normal morphology of the sciatic nerve of a mouse (Nguyen, *et al*., 2012).

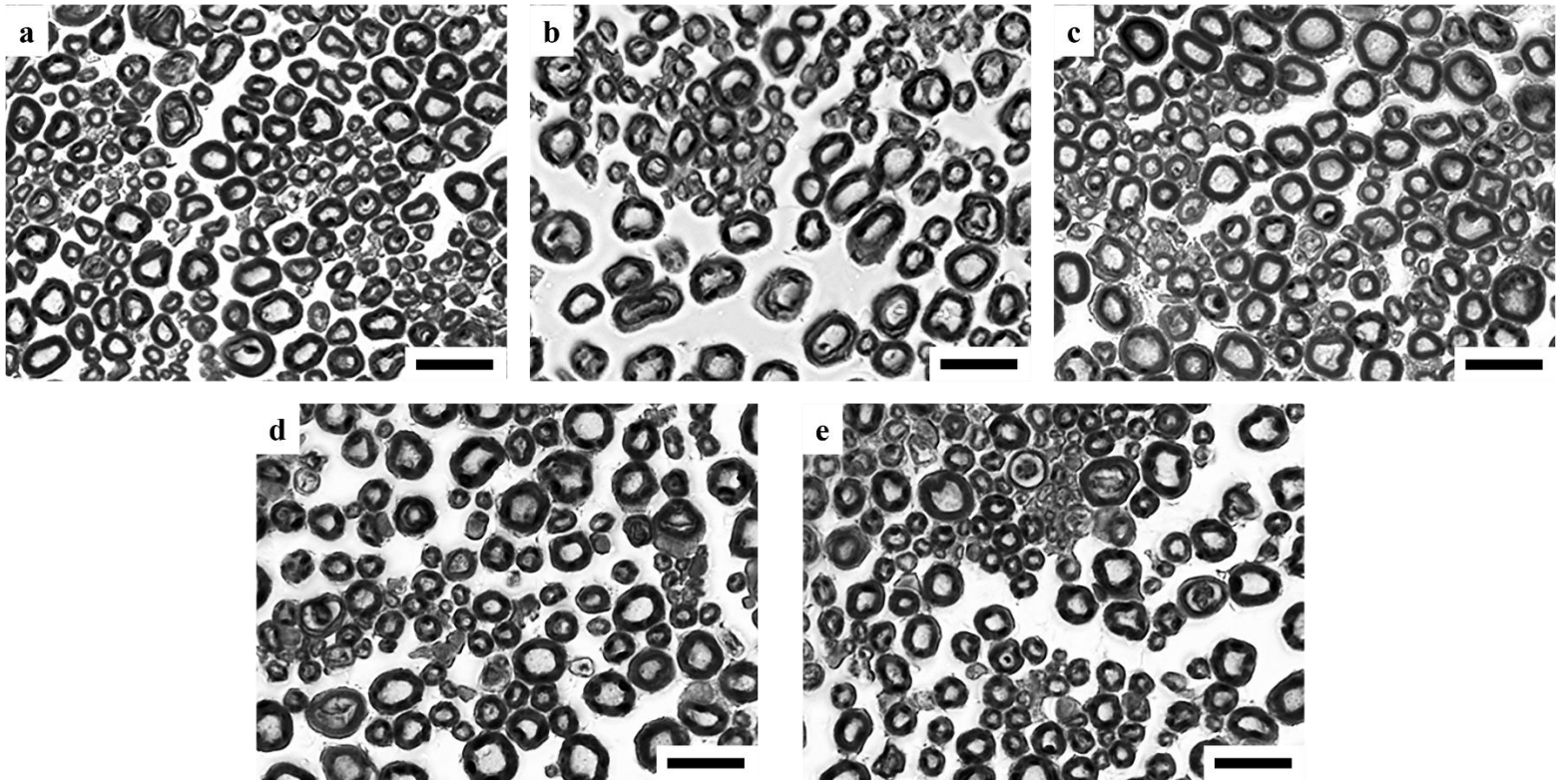


Figure 1: Representative photomicrographs of the sciatic nerve from the **a**) NT, **b**) SIM, **c**) ALC, **d**) ALC+SIM5, and **e**) ALC+SIM15 experimental groups. The axons were round to oval in shape and the dark rings represented the myelin sheath with its distinct inner and outer boundaries. The axons in all the different experimental groups appeared similar and typical of the normal histology of the sciatic nerve of mice. NT = Non-treatment; SIM = 5 mg Simvastatin; ALC = Alcohol; ALC+SIM5 = 5 mg Simvastatin and alcohol; ALC+SIM15 = 5 mg Simvastatin and alcohol. Scale bar = 10 µm.

### 3.1.2. Average axon density

The average axon density of the sciatic nerve across the different experimental groups and for both sexes is shown in Table 3. To reiterate, the average axon density was determined by counting the number of axons per counting grid for each image assessed. For the female mice, the average axon density was highest in the ALC+SIM5 and was lowest in the ALC. An ANOVA test revealed that the axon density was significantly different across the experimental groups ($P<0.001$). A Tukey's post hoc revealed that the average axon density was significantly higher in the NT than in the ALC ($P=0.003$) – an indication of alcohol toxicity on the density of axons. Similarly, the average axon density in the ALC+SIM5 was significantly higher than in the SIM ($P=0.029$), the ALC ($P<0.001$), or the ALC+SIM15 ($P=0.017$). However, there was no significant difference in the average axon density between the NT and the ALC+SIM5 ($P=0.980$) or between the SIM and the ALC ($P=0.726$). These results showed that the administration of 5 mg (but not 15 mg) Simvastatin improved the density of axons against the effect of alcohol in the females.

For the male mice, the average axon density was highest in the NT and lowest in the ALC groups. A Kruskal-Wallis test revealed that the average axon density was also significantly different across the experimental groups ($P=0.007$). In contrast to the observations in the females, a Dunn's test revealed that the axon density in the NT was significantly higher than in the SIM ($P=0.016$), the ALC ($P=0.0003$), the ALC+SIM5 ($P=0.008$) or the ALC+SIM15 ($P=0.011$) in the males. Interestingly, the average axon density in the males was similar in the SIM, ALC, ALC+SIM5, and ALC+SIM15. These results also confirmed that alcohol reduced axon density in the males but there seems to be no beneficial effect of Simvastatin against the effect of alcohol in the males. Comparing these observations with that of the female mice, it seems to show that the effect of Simvastatin against alcohol effect was sex–specific thus highlighting a possible influence of an underlying biological factor.

### 3.1.3. Average normalized myelin thickness

The average normalized values for the myelin thickness (i.e., raw and normalized) for all the experimental groups and both sexes are shown in Table 3. The obtained value of the myelin thickness of an axon was normalized by dividing the raw value of myelin thickness by the area of the corresponding nerve fibre. For the female mice, the average normalized myelin thickness was highest in the SIM while the ALC+SIM15 had the lowest. A Kruskal–Wallis test revealed a significant difference in the average normalized myelin thickness across the experimental groups ($P<0.001$). A Dunn's test showed that the average normalized myelin thickness in the NT was significantly higher than in the SIM ($P<0.001$), the ALC ($P<0.001$), the ALC+SIM5

($P<0.001$), or the ALC+SIM15 ($P<0.001$). In addition, the average normalized myelin thickness was significantly reduced in the ALC than in the NT ($P<0.001$), the SIM ($P=0.004$), the ALC+SIM5 ($P<0.001$), or the ALC+SIM15 ($P<0.001$). These findings also indicate that myelination was significantly reduced by alcohol. The effect of Simvastatin against alcohol effect was similar for the two concentrations of Simvastatin and they significantly improved myelination against the effect of alcohol.

For the males, the average normalized myelin thickness was highest in the SIM while the ALC+SIM5 had the lowest. There was a significant difference in the average normalized myelin thickness across the experimental groups ($P<0.001$). A Dunn's test showed that the average normalized myelin thickness in the NT was also significantly higher than in the SIM ($P=0.000$), the ALC ($P<0.001$), the ALC+SIM5 ($P<0.001$) or the ALC+SIM15 ($P<0.001$). Similar to the females, the average normalized myelin thickness was also significantly reduced in the ALC than in the NT ($P=0.000$), the SIM ($P<0.001$) or the ALC+SIM5 ($P<0.001$) but was similar in the ALC+SIM15 ($P=0.934$). Interestingly, the average normalized myelin thickness was significantly higher in the ALC+SIM5 than in the ALC+SIM15 ($P<0.001$). These results showed that alcohol had a damaging effect on the myelin thickness of the sciatic nerve. At the same time, both concentrations of Simvastatin had a protective effect against the damaging effects of alcohol except in the males where the high concentration (i.e., 15 mg) was not effective. This is an indication of sex–specific differences in the effect of Simvastatin which may also highlight a possible influence of an underlying biological factor with respect to myelination.

Table 4: An illustration showing the average values of the axon density, myelin thickness, and g–ratio for all the experimental groups for both sexes

| | Axon density | | | Myelin thickness | | | | | g–ratio | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Group | The total no of grids assessed | Average (count/µm²) ($10^{-4}$) | SD (count/µm²) ($10^{-4}$) | The total no of axons assessed | Average (Raw) (µm) | SD (Raw) (µm) | Average (Normalized) ($10^{-5}$) | SD Normalized ($x10^{-5}$) | The total no of axons assessed | Average | SD |
| **Female** | | | | | | | | | | | |
| NT | 42 | 4.516 | 0.876 | 300 | 2.335 | 0.649 | 14.700 | 13.558 | 300 | 0.56 | 0.10 |
| SIM | 35 | 3.331 | 0.41 | 150 | 2.443 | 0.627 | 7.862 | 2.741 | 150 | 0.57 | 0.10 |
| ALC | 62 | 2.674 | 1.124 | 200 | 2.304 | 0.955 | 6.841 | 4.683 | 200 | 0.59 | 0.11 |
| ALC+SIM5 | 78 | 4.763 | 1.759 | 350 | 2.291 | 1.059 | 9.358 | 6.346 | 350 | 0.62 | 0.09 |
| ALC+SIM15 | 66 | 3.496 | 1.075 | 350 | 2.075 | 0.731 | 9.870 | 6.220 | 350 | 0.59 | 0.11 |
| **Male** | | | | | | | | | | | |
| NT | 39 | 4.578 | 0.982 | 200 | 2.171 | 0.654 | 19.350 | 12.929 | 200 | 0.58 | 0.10 |
| SIM | 77 | 3.596 | 1.371 | 400 | 2.378 | 0.753 | 11.110 | 3.987 | 400 | 0.56 | 0.11 |
| ALC | 89 | 2.967 | 1.166 | 250 | 2.223 | 0.755 | 7.551 | 4.459 | 250 | 0.59 | 0.11 |
| ALC+SIM5 | 72 | 3.293 | 1.227 | 300 | 1.992 | 0.620 | 10.783 | 4.998 | 300 | 0.61 | 0.10 |
| ALC+SIM15 | 75 | 3.413 | 1.341 | 250 | 2.125 | 0.678 | 7.860 | 5.165 | 250 | 0.59 | 0.09 |

SD = Standard deviation

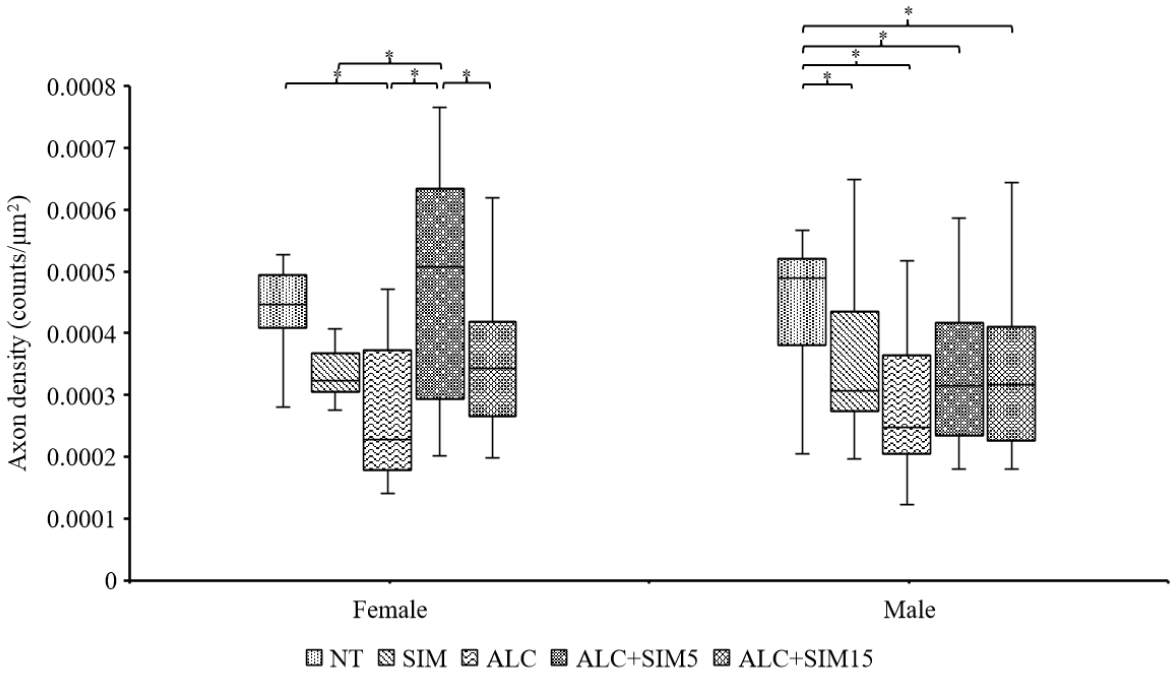


Figure 2: Box plots showing the average axon density of the sciatic nerve across the experimental groups in the female and the male. For both sexes, the average axon density was significantly lower in the ALC than in the NT. This is an indication of alcohol toxicity on axonal density. Only 5 mg Simvastatin was effective against the effect of alcohol in the females. However, both concentrations were not beneficial against the effect of alcohol in the males. This highlights possible sex–specific differences in the effect of Simvastatin against the alcohol effect. NT = Non-treatment; SIM = 5 mg Simvastatin; ALC = Alcohol; ALC+SIM5 = 5 mg Simvastatin and alcohol; ALC+SIM15 = 5 mg Simvastatin and alcohol. *$P$<0.05.

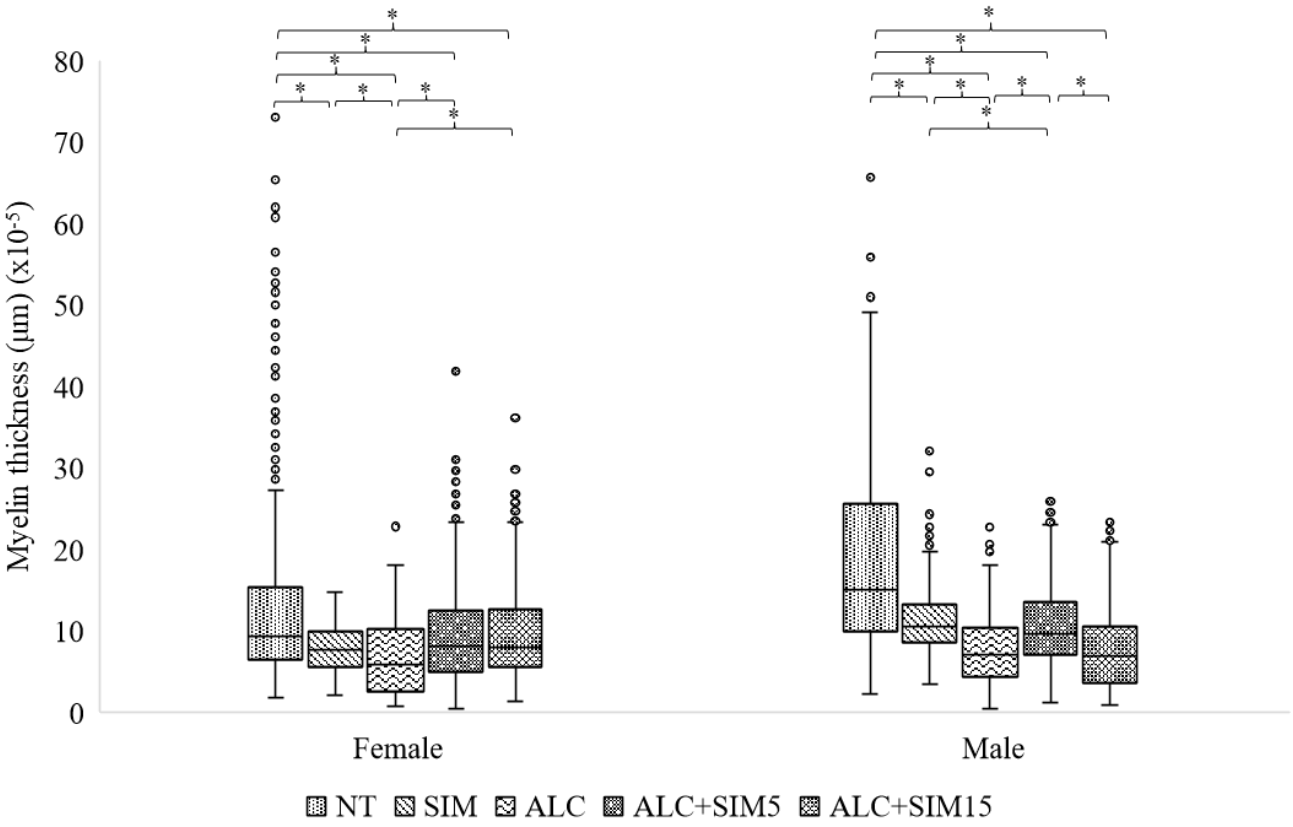


Figure 3**:** Box plots showing the average normalized myelin thickness of the sciatic nerve across the experimental groups in the female and the male. For both sexes, the average normalized myelin thickness was significantly higher in the NT than in the SIM, the ALC, the ALC+SIM5, or the ALC+SIM15. This indicates alcohol toxicity on myelination. Both concentrations of Simvastatin were effective against the alcohol effect on myelination. NT = Non-treatment; SIM = 5 mg Simvastatin; ALC = Alcohol; ALC+SIM5 = 5 mg Simvastatin and alcohol; ALC+SIM15 = 15 mg Simvastatin and alcohol. * $P<0.05$

### 3.1.4. Patterns of distribution of the g–ratio

The average g–ratio for each experimental group is shown in Table 3. The range of the g–ratio was similar for both sexes (range = 0.56 – 0.62). To further give a clearer understanding of the axonal g–ratios and their integrities, the g–ratios were categorized into three groups i.e., Group I, II, and III (Ugrenovic et al., 2014). The percentage distribution of the g–ratio in the different categories for all the experimental groups and according to sex are shown in Fig. 4. In the females, most axons in the NT (60%) and SIM (60%) had a g–ratio lower than 0.6 (Group I) compared to the axons in the ALC (51%), the ALC+SIM5 (34%) or the ALC+SIM15 (44%). This seems to indicate that these axons (i.e., Group 1 axons) were probably newly regenerated axons with relatively thick myelin. It is also possible that the administration of simvastatin did not 'majorly' promote axonal regeneration in the females. The males also exhibited a similar pattern in that the percentage distribution of the Group I axons was in the range of 41 – 58% with the ALC+SIM5 having the lowest percentage distribution of Group I axons.

For the percentage distribution of Group II axons in the females, the distribution was within the same range in the NT (35%), the SIM (33%) and the ALC (32%) but was slightly higher in the ALC+SIM5 (48%) and the ALC+SIM15 (41%). This seems to show that the distribution of axons with an optimal g–ratio was improved by Simvastatin in the presence of alcohol effect. On the other hand, the percentage distribution of Group II axons in the males was highest in the NT (44%) and the distributions in the other experimental groups were within the same range (i.e., SIM = 36%, ALC = 32%, ALC+SIM5 = 38%, ALC+SIM15 = 37%). This also seems to show that the administration of Simvastatin also promoted the distribution of axons with an optimal g–ratio.

The percentage distribution of Group III axons was identical in both sexes with a range of 5 – 17% in the females and 6 – 21% in the males across the experimental groups. This is an interesting observation in that the NT in the females and the SIM in the males had the lowest distributions of degenerative (i.e., Group III) axons compared to the other experimental groups that were administered alcohol (i.e., ALC, ALC+SIM5, and ALC+SIM15). This also indicates that alcohol caused degeneration of axons (i.e. thin myelin) and that the two concentrations of Simvastatin did not have an 'overwhelming' beneficial effect against the damaging effects of alcohol in both sexes.

Figure 5 further shows the characteristics of the data concerning the patterns of distribution of the g–ratio in both sexes. From the graphs, it is apparent that most of the axons have a g–ratio

within 0.6 – 0.7 across the experimental groups, and there were smaller peaks for the g–ratio less than 0.6 compared to the steep slopes for the g–ratio greater than 0.7 across the experimental groups.

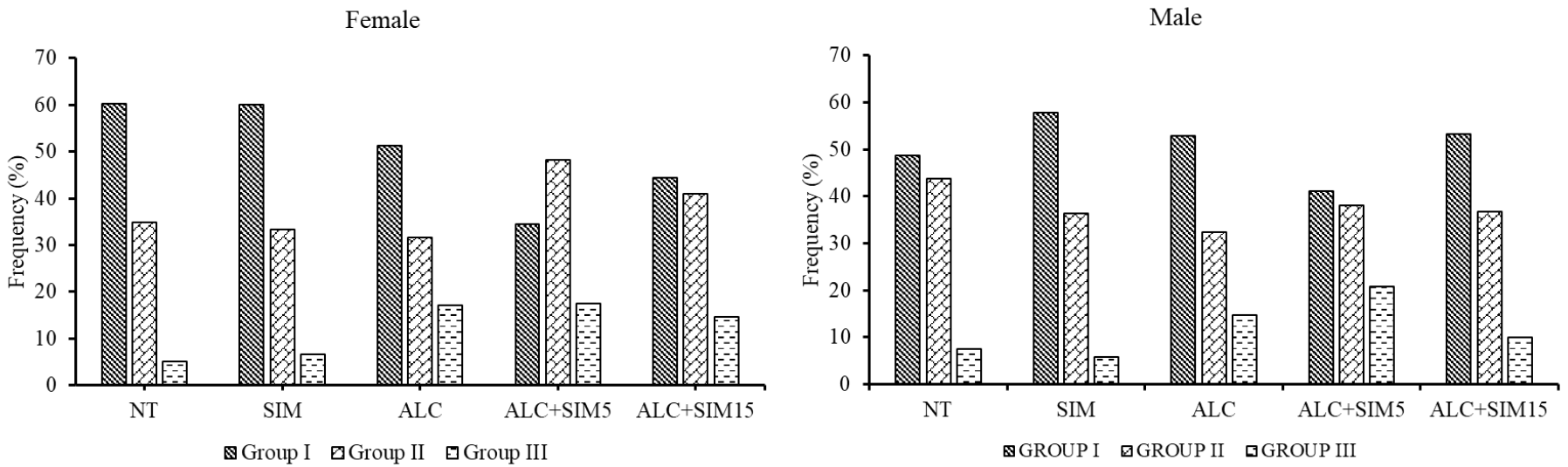


Figure 4**:** Bar graphs showing the percentage frequency of g–ratios categorised into groups. Group I (g–ratio <0.6), Group II (g–ratio = 0.6 – 0.7), and Group III (g–ratio > 0.7). NT = Non-treatment; SIM = 5 mg Simvastatin; ALC = Alcohol; ALC+SIM5 = 5 mg Simvastatin and alcohol; ALC+SIM15 = 15 mg Simvastatin and alcohol.

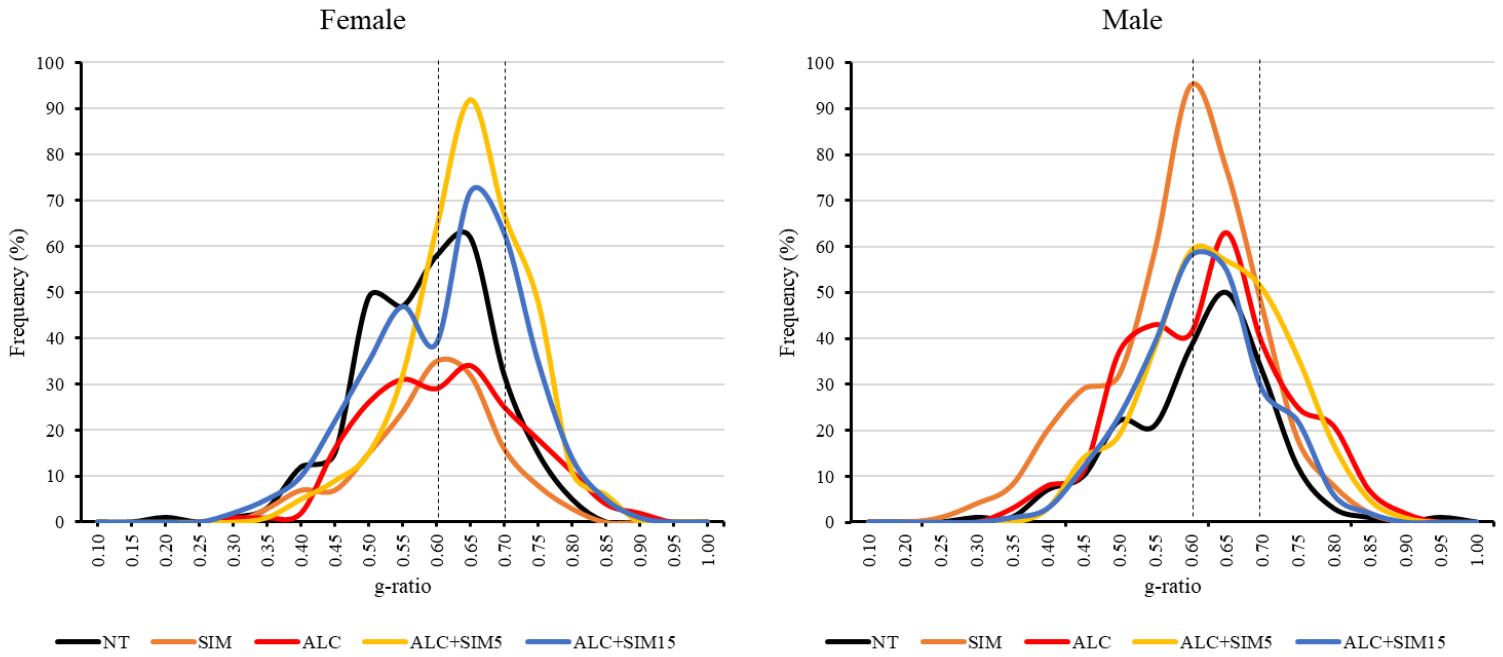


Figure 5: Frequency plots of the distribution of the g–ratio of axons across the different experimental groups in both sexes. In both sexes, axons with optimal g–ratios (0.6 – 0.7) were most abundant. Interestingly, the distribution of axons with g–ratios of <0.6 (indicated by less steep curves with remarkable or obvious peaks) were also more abundant than those axons with g–ratio >0.7 (indicated by steep curves with less remarkable or obvious peaks) for all the experimental groups in both sexes. NT = Non-treatment; SIM = 5 mg Simvastatin; ALC = Alcohol; ALC+SIM5 = 5mg Simvastatin and alcohol. ALC+SIM15= 15mg Simvastatin and alcohol.

### 3.5 Morphometry of PMBSF barrels

#### 3.5.1. General appearance and organisation of PMBSF barrels

All the PMBSF barrels were present in all the mice used in the study. The PMBSF barrels appeared CO–reactive (i.e., dark stained) with no distorted barrel boundaries. In addition, the PMBSF barrel organization was similar in all the mice across the different experimental groups and their appearance was typical of those reported in other studies (e.g., Woosley and Van der Loos, 1970; Olateju *et al*., 2019). Similar to a typical barrel organization, the PMBSF barrels in the present study were easily identified as consisting of five distinct barrel rows (A – E) with 4 barrels in rows A and B and 5 barrels in rows C – E as well as having four distinct large straddler barrels (α-δ) on the posteromedial border of the PMBSF. A representative photomicrograph of the PMSBF organization as well as the nomenclature used in describing the barrels is shown in Figures 6a and b.

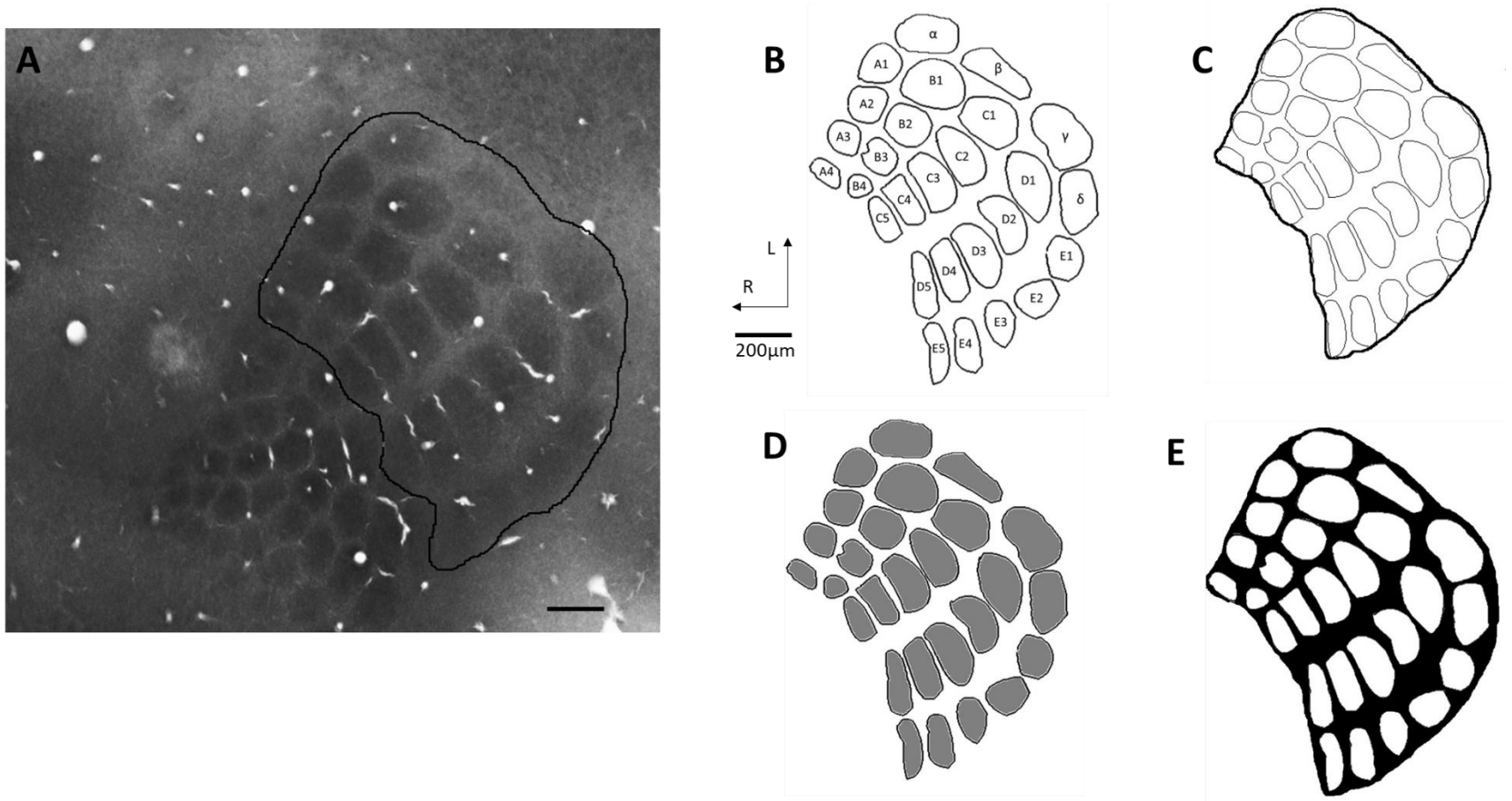


FIGURE 6 **(A-E):** Diagrams indicating (**A**) a photomicrograph of the posterior-medial barrel subfield (PMBSF) barrels and outline of the PMBSF enclosure from which measurements were taken; the white circles in the image are the blood vessels (scale bar = 200 µm), (**B**) the nomenclature used for the barrels within the PMBSF, (**C**) the measurement made to calculate the area of the enclosure of the PMBSF, (**D**) the measurements made to obtain data for individual barrel areas, and when combined the total area of the PMBSF, and (**E**) the measurement made to define the septal area of the PMSBF

### 3.5.1 Total average area of the PMSBF

The summary of the morphometries of the PMBSF barrels for all the measurements and for both sexes is shown in Table 6. The total area of the PMBSF barrels was determined by adding the areas of the 27 individual barrels of each mouse. The total average area of the PMBSF was lowest in the SIM in the females while the ALC+SIM15 was the lowest in the males. Interestingly, an ANOVA test revealed that the decrease in the total average area of the PMBSF was not statistically significant across the experimental groups in the females ($P$=0.201) or in males ($P$=0.317). These results revealed no damaging effect of alcohol on the total average area of the PMBSF barrels in both sexes (Fig 7A).

### 3.5.2 Average area of the PMSBF enclosure

The average area of the barrel enclosure in the ALC+SIM5 was highest in the females, and the NT in the males recorded the highest (Table 6). The average area of the PMBSF enclosures was not statistically significantly different across the experimental groups in the females ($P$=0.097) and in the male ($P$=0.922) according to an ANOVA test. These results show that alcohol or Simvastatin did not impact the size of the PMBSF enclosure (Fig 7B).

### 3.5.3 Average area of the septal portion of the PMBSF

The average area of the septal portion of the PMBSF was determined by subtracting the total area of the individual PMBSF barrels from the area of the PMBSF barrel enclosure. The obtained values for both sexes across the experimental groups are shown in Table 6. In the females, the average area of the septal portion of the PMBSF was lowest in the ALC and the lowest was in the NT in the males. An ANOVA test revealed that the total average septal portion of the PMBSF was significantly different across the experimental groups in the females ($P$=0.009). A Tukey's test revealed that the average area of the septal portion of the PMBSF in the ALC was significantly reduced compared to that of the SIM ($P$=0.028) or the ALC+SIM5 ($P$=0.005) but was not significantly different from the NT ($P$=0.137) or the ALC+SIM15 ($P$=0.343). Alcohol was also not neurotoxic to the septal portion of the PMBSF because the size of the septal portion of PMBSF for the ALC and NT were similar. There was also no significant difference in the average area of the septal portion of the PMBSF across the experimental groups in the males ($P$=0.985) (Fig 7C).

### 3.5.4. Average areas of the individual PMSBF barrels

The average area of the individual PMBSF barrel was determined by averaging the total area of the 27–PMBSF barrels. Similar to the results of the total average area of the PMBSF barrels or the average area of PMBSF enclosure, the average area of the individual PMBSF barrels was also not significantly different across the experimental groups in the females ($P$=0.201) or in the males ($P$= 0.317) according to an ANOVA test. These results show that alcohol or Simvastatin did not also influence the size of the individual PMBSF barrels (Fig 7D).

Table 5: A table showing the mean values of the measured parameters across the experimental groups for the PMBSF barrels for each sex using one-way ANOVA

| Experimental group | Sample size | The total average area of PMBSF | | The average area of individual PMBSF barrels | | Average PMBSF enclosure area | | Average septal area of PMBSF | |
|---|---|---|---|---|---|---|---|---|---|
| | | Mean ($mm^2$) | SD ($mm^2$) | Mean ($mm^2$) | SD ($mm^2$) | Mean ($mm^2$) | SD ($mm^2$) | Mean ($mm^2$) | SD ($mm^2$) |
| **Female** | | | | | | | | | |
| NT | 3 | 0.486 | 0.052 | 0.018 | 0.002 | 0.866 | 0.076 | 0.38 | 0.026 |
| SIM | 3 | 0.409 | 0.022 | 0.015 | 0.001 | 0.827 | 0.042 | 0.418 | 0.042 |
| ALC | 3 | 0.532 | 0.064 | 0.020 | 0.002 | 0.813 | 0.079 | 0.291 | 0.017 |
| ALC+SIM5 | 4 | 0.523 | 0.098 | 0.019 | 0.004 | 0.970 | 0.084 | 0.456 | 0.067 |
| ALC+SIM15 | 3 | 0.494 | 0.030 | 0.018 | 0.001 | 0.871 | 0.072 | 0.38 | 0.051 |
| **Male** | | | | | | | | | |
| NT | 3 | 0.496 | 0.069 | 0.018 | 0.003 | 0.825 | 0.021 | 0.329 | 0.056 |
| SIM | 4 | 0.439 | 0.066 | 0.016 | 0.002 | 0.782 | 0.149 | 0.343 | 0.097 |
| ALC | 4 | 0.431 | 0.021 | 0.016 | 0.001 | 0.771 | 0.051 | 0.363 | 0.043 |
| ALC+SIM5 | 3 | 0.452 | 0.044 | 0.017 | 0.002 | 0.820 | 0.145 | 0.342 | 0.138 |
| ALC+SIM15 | 4 | 0.405 | 0.057 | 0.015 | 0.002 | 0.766 | 0.116 | 0.356 | 0.079 |

SD = Standard deviation

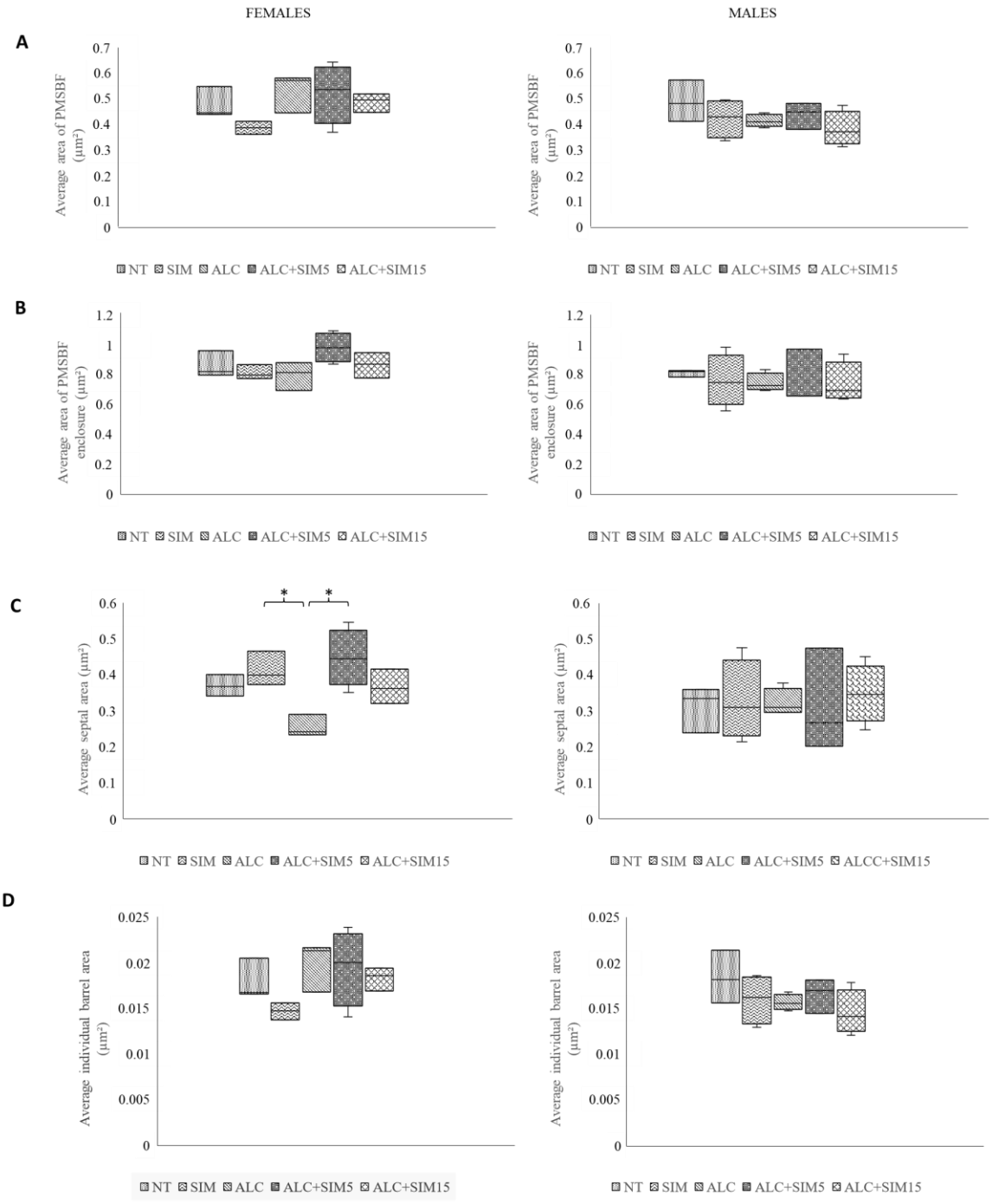


Figure 7 (**A**-**D**): Bar graphs showing the (**A**) Average total area of PMSBF barrels, (**B**) Average area of the PMSBF enclosure, (**C**) Average septal area (**D**) and Average individual PMSBF barrel area across the experimental groups in both sexes. The average total area of PMSBF barrels, the average area of the PMSBF enclosure, and the average individual PMSBF barrel area were insignificantly different in both sexes. The average septal area in the female mice was significantly lower in the ALC than in the SIM or ALC+SIM5.In the males, the average septal area was insignificantly different. NT = Non-treatment; SIM = 5 mg Simvastatin; ALC = Alcohol; ALC+SIM5 = 5 mg Simvastatin and alcohol; ALC+SIM15= 15mg Simvastatin and alcohol.

### 3.5.5. Barrel-to-barrel comparison

To further understand how individual barrel compares across the experimental groups, a barrel-to-barrel comparison was performed for all 27–PMBSF barrels for both sexes (Table 7; Fig. 8). For the females, an ANOVA test revealed no significant difference in the average area of the individual barrel in the rows B – E and the straddler barrels except for the barrels A2 ($P$=0.003) and A3 ($P$=0.007) where the average size of the A2 or A3 barrel in the ALC was significantly higher than in the other experimental groups [for the A2 barrel: $P_{NT}$=0.024, $P_{SIM}$=0.003), $P_{ALC+SIM5}$=0.005 or $P_{ALC+SIM15}$= 0.014; and for the A3 barrel: $P_{NT}$=0.028, $P_{SIM}$=0.017, $P_{ALC+SIM5}$=0.012 or $P_{ALC+SIM15}$= 0.010].

For the males, an ANOVA test revealed no significant difference in the average area of individual barrels in rows B, C, and the straddler barrels except for barrels A1 ($P$=0.003), D5 ($P$=0.011), and E2 ($P$=0.002). In the case of the A1 barrel, Tukey's test revealed that the average size of the A1 barrel in the ALC was significantly higher than in the SIM ($P$=0.046) or the ALC+SIM15 ($P$=0.001). In contrast, the average size of the D5 barrels in the ALC was significantly lower than in the other experimental groups ($P_{NT}$=0.030, $P_{SIM}$=0.044, $P_{ALC+SIM5}$=0.041 or $P_{ALC+SIM15}$=0.013) while the average size of the E2 barrels in the ALC was significantly lower than in the NT ($P$=0.004) or the ALC+SIM5 ($P$=0.004).

Despite the non-significant differences in the average sizes of all the individual PMBSF barrels or the total average area of the PMBSF barrels, barrel-to-barrel comparisons revealed alcohol toxicity on specific barrels. This seems to show that the effect of alcohol was barrel specific as in the case of the present study.

Table 6: A table showing the barrel-to-barrel comparisons of the average size of individual PMBSF barrels across the experimental groups for both sexes.

| | **Ave. area of individual barrels ($mm^2$)** | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **NT** | | **SIM** | | **ALC** | | **ALC+SIM5** | | **ALC+SIM15** | | ***P*** | |
| **Barrel** | **F** | **M** | **F** | **M** | **F** | **M** | **F** | **M** | **F** | **M** | **F** | **M** |
| **alpha** | 0.025 | 0.024 | 0.021 | 0.018 | 0.022 | 0.019 | 0.023 | 0.023 | 0.017 | 0.017 | 0.71 | 0.07 |
| **beta** | 0.030 | 0.027 | 0.020 | 0.026 | 0.024 | 0.019 | 0.028 | 0.023 | 0.027 | 0.022 | 0.57 | 0.41 |
| **gamma** | 0.028 | 0.028 | 0.020 | 0.026 | 0.027 | 0.017 | 0.027 | 0.019 | 0.024 | 0.019 | 0.59 | 0.10 |
| **delta** | 0.021 | 0.021 | 0.017 | 0.024 | 0.028 | 0.015 | 0.021 | 0.028 | 0.020 | 0.016 | 0.74 | 0.41 |
| **A1** | 0.014 | 0.015 | 0.013 | 0.012 | 0.021 | 0.020 | 0.011 | 0.013 | 0.015 | 0.008 | 0.21 | **0.00*** |
| **A2** | 0.012 | 0.012 | 0.009 | 0.011 | 0.019 | 0.018 | 0.010 | 0.010 | 0.011 | 0.008 | **0.00*** | 0.07 |
| **A3** | 0.009 | 0.012 | 0.008 | 0.009 | 0.018 | 0.014 | 0.008 | 0.006 | 0.007 | 0.010 | **0.01*** | 0.09 |
| **A4** | 0.005 | 0.009 | 0.005 | 0.008 | 0.017 | 0.011 | 0.007 | 0.006 | 0.007 | 0.006 | 0.06 | 0.16 |
| **B1** | 0.021 | 0.022 | 0.019 | 0.020 | 0.025 | 0.020 | 0.026 | 0.018 | 0.016 | 0.018 | 0.28 | 0.71 |
| **B2** | 0.018 | 0.019 | 0.015 | 0.018 | 0.024 | 0.014 | 0.016 | 0.013 | 0.019 | 0.013 | 0.32 | 0.58 |
| **B3** | 0.016 | 0.016 | 0.012 | 0.016 | 0.020 | 0.014 | 0.014 | 0.011 | 0.018 | 0.013 | 0.10 | 0.58 |
| **B4** | 0.007 | 0.006 | 0.006 | 0.006 | 0.017 | 0.009 | 0.008 | 0.007 | 0.008 | 0.007 | 0.25 | 0.92 |
| **C1** | 0.025 | 0.028 | 0.020 | 0.023 | 0.029 | 0.022 | 0.029 | 0.022 | 0.025 | 0.019 | 0.39 | 0.12 |
| **C2** | 0.021 | 0.021 | 0.017 | 0.021 | 0.024 | 0.017 | 0.022 | 0.022 | 0.021 | 0.018 | 0.41 | 0.54 |
| **C3** | 0.020 | 0.020 | 0.013 | 0.016 | 0.019 | 0.016 | 0.015 | 0.017 | 0.023 | 0.019 | 0.10 | 0.60 |
| **C4** | 0.014 | 0.014 | 0.014 | 0.016 | 0.019 | 0.015 | 0.021 | 0.014 | 0.017 | 0.011 | 0.59 | 0.33 |
| **C5** | 0.015 | 0.013 | 0.012 | 0.009 | 0.014 | 0.012 | 0.015 | 0.012 | 0.014 | 0.011 | 0.91 | 0.25 |
| **D1** | 0.026 | 0.024 | 0.019 | 0.019 | 0.024 | 0.023 | 0.024 | 0.024 | 0.024 | 0.021 | 0.77 | 0.59 |
| **D2** | 0.020 | 0.020 | 0.015 | 0.020 | 0.023 | 0.018 | 0.021 | 0.019 | 0.022 | 0.019 | 0.12 | 0.99 |
| **D3** | 0.019 | 0.022 | 0.017 | 0.017 | 0.020 | 0.017 | 0.019 | 0.017 | 0.020 | 0.017 | 0.88 | 0.74 |
| **D4** | 0.017 | 0.016 | 0.014 | 0.016 | 0.009 | 0.014 | 0.021 | 0.017 | 0.020 | 0.016 | 0.30 | 0.95 |
| **D5** | 0.016 | 0.016 | 0.013 | 0.014 | 0.005 | 0.004 | 0.023 | 0.015 | 0.017 | 0.016 | 0.06 | **0.01*** |
| **E1** | 0.022 | 0.017 | 0.021 | 0.018 | 0.021 | 0.016 | 0.019 | 0.020 | 0.019 | 0.018 | 0.82 | 0.67 |
| **E2** | 0.016 | 0.021 | 0.019 | 0.016 | 0.018 | 0.011 | 0.022 | 0.021 | 0.022 | 0.018 | 0.82 | **0.00*** |
| **E3** | 0.018 | 0.018 | 0.019 | 0.016 | 0.013 | 0.010 | 0.024 | 0.018 | 0.023 | 0.016 | 0.30 | 0.09 |
| **E4** | 0.015 | 0.019 | 0.017 | 0.012 | 0.011 | 0.009 | 0.021 | 0.013 | 0.018 | 0.015 | 0.15 | 0.06 |
| **E5** | 0.016 | 0.015 | 0.014 | 0.014 | 0.012 | 0.009 | 0.020 | 0.011 | 0.019 | 0.014 | 0.14 | 0.23 |

*Statistically significant at $P<0.05$

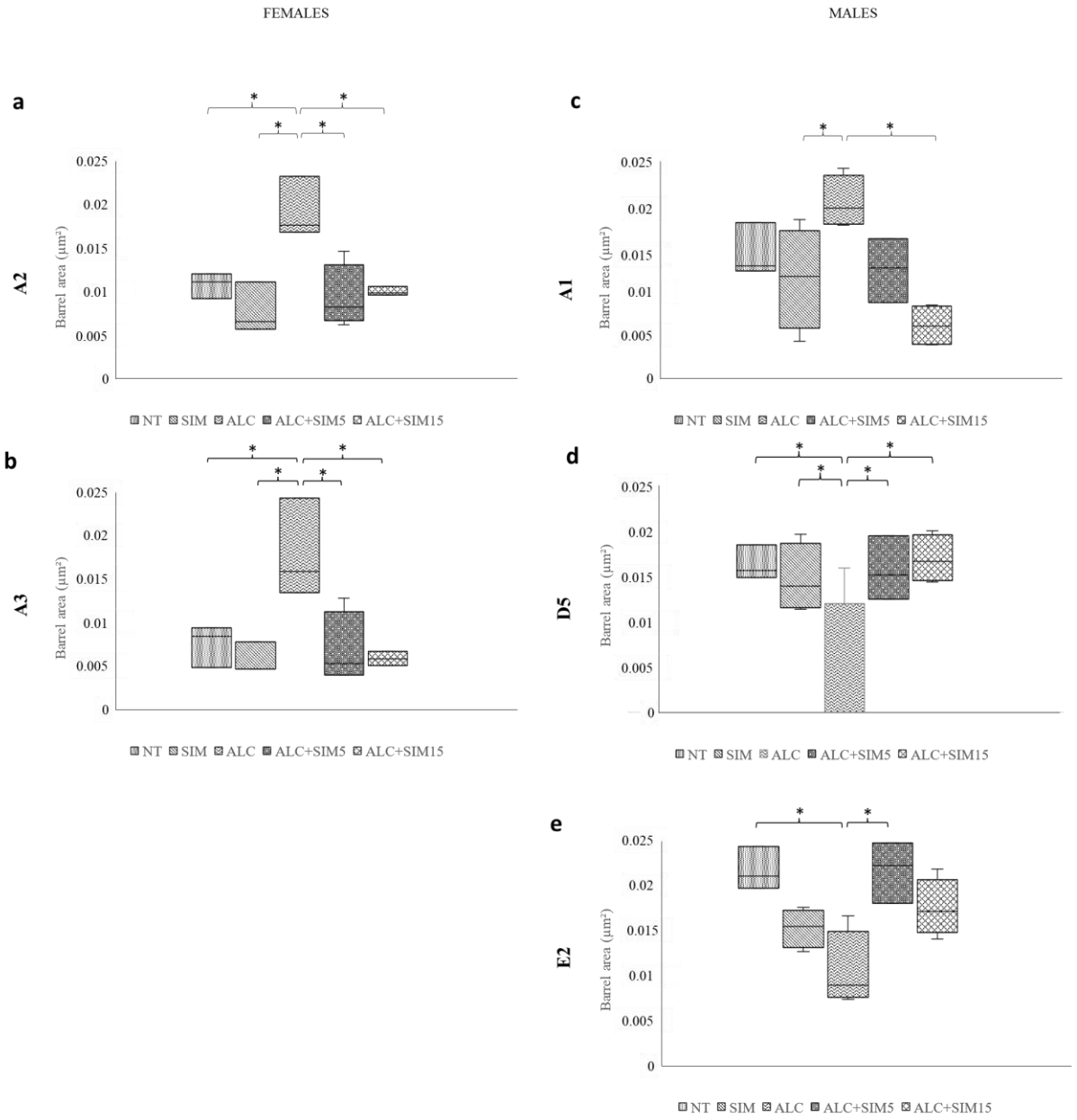


Figure 8: Box plots showing the **a**) average A2 and b) A3 barrel areas across the experimental groups in the female as well as **c**) the average A1, **d**) D5 and **e**) E2 barrel areas across the experimental groups in the male mice. The A2 or A3 barrel in the ALC was significantly higher than in the other experimental groups in the females whilst in the males, the A1 barrel area in the ALC was significantly higher than in the SIM or ALC+SIM5. The D5 barrel area in the ALC was significantly lower than in other experimental groups. The E2 barrel area in the ALC was significantly lower than in the NT or ALC+SIM5. NT = Non-treatment; SIM = Simvastatin; ALC = Alcohol; ALC+SIM5 = 5 mg Simvastatin and alcohol; ALC+SIM15 =5mg Simvastatin and alcohol. *P<0.05

### 3.5.6. Comparisons of barrels in rows and arc

To further provide more details on the comparisons of individual PMBSF barrels, the average area of all the barrels in the same row or arc was compared across the different experimental groups (Table 8). An ANOVA test revealed that there was a significant difference in the average area of the barrels in row A in the females ($P$=0.001) and the males ($P$=0.014). In the females, a Tukey's test revealed that the average area of the barrels in row A of the ALC was significantly higher than the other experimental groups ($P_{NT}$=0.004, $P_{SIM}$=0.002, $P_{ALC+SIM5}$=0.003 or $P_{ALC+SIM15}$=0.005) while the average barrel size in row A was significantly increased in the ALC than in the ALC+SIM5 ($P$=0.031) or ALC+SIM15 ($P$=0.014) in the males (Figure 9A).
In addition, an ANOVA test revealed that there was a significant difference in the average area of the barrels in row E in the females ($P$=0.032) and the males ($P$=0.014) mice. In the females, a Tukey's test revealed that the average area of the barrels in row E of the ALC was significantly lower than in the ALC+SIM5 ($P$=0.027) Interestingly, in the males, the average barrel size in row E was significantly lower in the ALC than the NT group ($P_{NT}$=0.008) (Figure 9B).

The observations in the female and male mice showed similar patterns in that similar rows were significantly affected by alcohol. It is however surprising to find that the effect of alcohol on the average size of barrels in the rows was significantly increased in row A but its effect was significantly reduced in row E.

In addition, a comparison of the PMSBF barrels in each arc was performed across all the experimental groups for both sexes (Table 8). In the female, only the barrels in Arc 1 produced a significant difference in the average barrel size in the arc across the experimental groups in the females ($P$=0.035). A Tukey's post hoc test revealed that the average barrel size in Arc 1 in the ALC was significantly lower than in the ALC+SIM5 ($P_{ALC+SIM5}$= 0.027). (Table 8; Figure 9D). In the males, only the straddler barrels produced a significant difference in the average barrel size in the arc across the experimental groups in the males ($P$=0.048). A Dunn's test revealed that the average barrel size in the straddler arc in the ALC was significantly lower than in the NT ($P$=0.036), SIM ($P$=0.03132), or ALC+SIM5 (P=0.036) (Table 8; Figure 9E). These observations revealed that the barrels in different arcs were also significantly affected by alcohol to a varying degree, thus indicating alcohol toxicity on the sizes of the barrel cortices.

Table 7: An illustration of the average area of all the barrels in the PMBSF rows and arcs for both sexes across the experimental groups.

| | **Ave. area of rows (mm²)** | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Rows | NT | | SIM | | ALC | | ALC+SIM5 | | ALC+SIM15 | | p-value | |
| | F | M | F | M | F | M | F | M | F | M | F | M |
| A | 0.010 | 0.012 | 0.009 | 0.010 | 0.019 | 0.016 | 0.009 | 0.009 | 0.010 | 0.008 | 0.001* | 0.014* |
| B | 0.016 | 0.016 | 0.013 | 0.015 | 0.021 | 0.017 | 0.015 | 0.013 | 0.015 | 0.013 | 0.284 | 0.746 |
| C | 0.019 | 0.019 | 0.015 | 0.017 | 0.021 | 0.016 | 0.020 | 0.017 | 0.020 | 0.016 | 0.363 | 0.870 |
| D | 0.019 | 0.019 | 0.016 | 0.017 | 0.016 | 0.016 | 0.020 | 0.019 | 0.021 | 0.018 | 0.291 | 0.706 |
| E | 0.017 | 0.018 | 0.018 | 0.015 | 0.015 | 0.011 | 0.021 | 0.017 | 0.020 | 0.016 | 0.032* | 0.014* |
| Arcs | **Ave. area of arcs (mm²)** | | | | | | | | | | | |
| 1 | 0.016 | 0.015 | 0.013 | 0.015 | 0.010 | 0.012 | 0.019 | 0.013 | 0.017 | 0.014 | 0.035* | 0.697 |
| 2 | 0.011 | 0.013 | 0.011 | 0.012 | 0.015 | 0.012 | 0.014 | 0.011 | 0.014 | 0.012 | 0.870 | 0.990 |
| 3 | 0.016 | 0.017 | 0.014 | 0.015 | 0.018 | 0.016 | 0.015 | 0.014 | 0.018 | 0.015 | 0.524 | 0.835 |
| 4 | 0.017 | 0.019 | 0.015 | 0.017 | 0.021 | 0.017 | 0.018 | 0.017 | 0.019 | 0.016 | 0.180 | 0.863 |
| 5 | 0.022 | 0.021 | 0.018 | 0.018 | 0.024 | 0.021 | 0.019 | 0.020 | 0.020 | 0.016 | 0.374 | 0.431 |
| Straddler | 0.024 | 0.024 | 0.020 | 0.023 | 0.025 | 0.018 | 0.025 | 0.023 | 0.022 | 0.018 | 0.179 | 0.048* |

F = Female
M = Male
*$P$<0.05

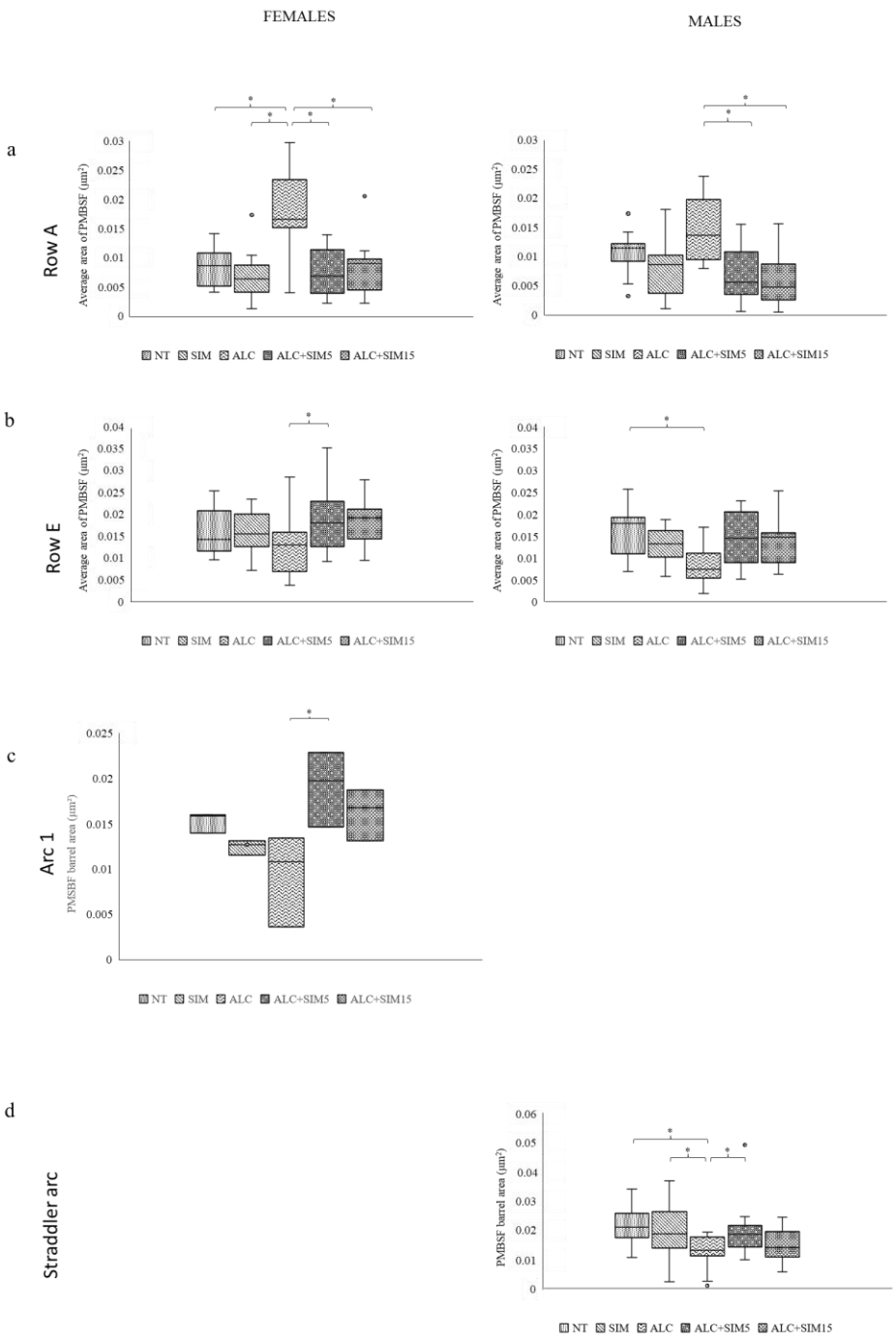


Figure 9: Boxplots of the average size of the PMBSF barrels in **a**) row A for both sexes, **b**) row E for both sexes, **c**) Arc 1 for the females **d**) and the straddler arc for the males across the different experimental groups. Alcohol–induced damage was specific to some PMBSF barrels in some rows (i.e. A and E) and arcs (arc 1 and straddler) and its toxic effect seem to be sex–specific. In addition, the effect of Simvastatin against alcohol toxicity was effective to varying degrees. NT = Non-treatment; SIM = 5 mg Simvastatin; ALC = Alcohol; ALC+SIM5 = 5 mg Simvastatin and alcohol; ALC+SIM15 = 5 mg Simvastatin and alcohol. *$P$<0.05

# CHAPTER 4

# DISCUSSION

## 4.1 Discussion

Adolescent alcoholism is a major problem as its toxic effects affect different parts of the brain thus contributing to the financial burden of the Government of South Africa (Matzopoulos et al., 2014). Despite the awareness, campaigns, and regulation of access to alcohol by the underage (Parry *et al*., 2012; Tanner-Smith & Lipsey, 2015), the prevalence of adolescents consuming alcohol is still on the rise (Peltzer *et al*., 2011; Morojele & Ramsoomar, 2016) which highlights the inefficiencies of these approaches. This has thus necessitated the exploration of interventions that could mask or prevent the damaging effects of alcohol. Simvastatin has proven promising due to its anti–oxidative and neuroprotective effects against neurodegeneration (van der Most *et al*., 2009; Wang *et al*., 2011; Chataway *et al*., 2014). The present study explored the neuroprotective effects of Simvastatin against alcohol–induced peripheral neuropathy and on the barrel sizes of the somatosensory cortices of mice. The BAC levels in the mice that were administered alcohol confirmed that a physiologically significant amount of alcohol was administered (Rhodes *et al*., 2005; Sulik, 2005) during the 4 weeks of treatment (i.e., from PND28 – 56).

## 4.2 Simvastatin significantly prevented alcohol-induced peripheral neuropathy

In the present, alcohol caused significant damage to the density of the axons in both sexes despite finding no observable differences in the morphologies of the sciatic nerves across the different experimental groups. The present findings are consistent with Nguyen *et al*. (2012) that reported a significant reduction in the average axonal density in the sciatic nerve of adult Long-Evans rats that were pair-fed with isocaloric liquid diets containing varying concentrations of ethanol for a period of 8 weeks. Similarly, other studies also reported a significant reduction in the axonal density following the administration of alcohol (e.g., Juntunen *et al*., 1978; Bosch *et al*., 1979; Mellion *et al*., 2013; Kashkin *et al*., 2016).

Other studies that investigated alcohol effects on other types of nerves also reported similar findings. For example, Igit and Colcimen (2019) reported that alcohol significantly reduced the axonal density of the optic nerve of rats following alcohol administration. Likewise, prenatal alcohol exposure was also reported to significantly reduce the axonal density of the optic nerve

of rats (Pinazo-Duran *et al*., 1993; Sawada *et al*., 2002) and in the axonal density of the sural nerve of adult human alcoholics (Walsh & McLeod, 1970; Koike *et al*., 2003). However, other studies reported no difference in the axonal density of the sciatic nerves of male Wistar rats that were administered alcohol for a period of 8 weeks (Tessitore et al., 2022) and in prenatally exposed rats to alcohol (Aktaş et al., 2009). Likewise, Komatsu *et al*. (2003) reported no significant difference in the axonal density in the facial nerves of rats that were also prenatally exposed to alcohol.

Similar to the axonal density, the myelin thickness of axons was also significantly reduced by alcohol in both sexes. This is also consistent with previous studies that reported that alcohol caused a significant decrease in the myelin thickness of axons in the sciatic nerve of adult Wistar rats that were exposed to alcohol for 8 weeks (Tessitore *et al*., 2022). Kahkin *et al*. (2016) equally reported a significant decrease in the myelin thickness of axons in the sciatic nerves of adult Wistar rats that were exposed to increasing concentrations of alcohol (7.47 to 26.2% ) for 15 weeks, as did Ertem *et al*. (2009) who reported a significant decrease in the myelin thickness of axons in the posterior tibial nerve of adult Wistar rats that were exposed to increasing concentrations of alcohol (2.4 to 7.4%) for 7 days (Ertem *et al*., 2009). Similarly, prenatal alcohol was teratogenic to the myelin thickness of the facial nerves of mice (Komatsu *et al*., 2003) and the sural nerves of adult human alcoholics (Koike *et al*., 2003). However, Juntunen *et al*. (1983) found no significant reduction in the myelin thickness of axons in the sciatic nerves of adult male mice exposed to alcohol. In addition, prenatal alcohol exposure was not teratogenic to the myelin thickness of the optic nerves of rats (Sawada *et al*., 2002).

The differences in the results between the present study and the other studies that reported no significant differences in the effects of alcohol on axonal density or myelin thickness may be due to differences in animal species [mouse (Juntunen et al., 1983; Dursun et al., 2013), rat (Bosch *et al*., 1979; Pinazo-Duran *et al*., 1993; Sawada *et al*., 2002; Komatsu *et al*., 2003; Aktaş *et al*., 2009; Pons-Vázquez *et al*., 2011; Nguyen *et al*., 2012; Mellion *et al*., 2013; Kashkin *et al*., 2016; Igit & Colcimen, 2019; Tessitore *et al*., 2022), or humans (Walsh & McLeod, 1970; Tackmann *et al*., 1977; Koike *et al*., 2003; Koike & Sobue, 2006)] or differences in methodologies such as the age of the animal [e.g., adult (Bosch *et al*., 1979; Juntunen *et al*., 1983; Ertem *et al*., 2009; Nguyen *et al*., 2012; Mellion *et al*., 2013; Kashkin *et al*., 2016; Igit & Colcimen, 2019; Tessitore *et al*., 2022) or adolescents (the present study)], the type of nerve [sciatic nerve (Bosch et al., 1979; Juntunen et al., 1983; Aktaş et al., 2009; Nguyen et al., 2012; Kashkin et al., 2016; Tessitore et al., 2022), tibial nerve (Ertem et al., 2009), optic nerve

(Pinazo-Duran *et al*., 1993; Sawada *et al*., 2002; Pons-Vázquez *et al*., 2011;Igit & Colcimen, 2019), sural nerve (Walsh & McLeod, 1970; Tackmann *et al*., 1977; Koike *et al*., 2003; Koike & Sobue, 2006), facial nerve (Komatsu et al., 2003)], the mode of alcohol administration [e.g., pair-fed (Nguyen et al., 2012), oral gavage (Dursun et al., 2013), *ad libitum* (Juntunen et al., 1983; Kashkin et al., 2016; Tessitore et al., 2022), liquid diet (Bosch et al., 1979; Sawada *et al*., 2002; Ertem *et al*., 2009; Pons-Vázquez *et al*., 2011; Mellion *et al*., 2013) or intraperitoneal injection (present study; Komatsu et al., 2003; Aktaş et al., 2009)], prenatal (Pinazo-Duran *et al*., 1993; Sawada *et al*., 2002; Komatsu *et a*l., 2003; Aktaş *et al*., 2009; Pons-Vázquez *et al*., 2011) or postnatal period (Pinazo-Duran et al., 1993; Pons-Vázquez et al., 2011 Dursun et al., 2013). These factors have been highlighted to have an implication on the outcomes of test substances using experimental animals (Webster *et al*., 1983; Hayashi *et al*., 1989; Demetrius, 2005; Jackson *et al*., 2017; Arias-Reyes *et al*., 2021; Kuhns *et al*., 2022).

Alcohol–induced reductions in axonal density and myelin thickness observed in the present study may be have been caused by the neurodegenerative effects of alcohol and these observations are consistent with many studies on the peripheral and cranial nerves that have demonstrated the neurotoxic effects of alcohol on different types of nerves and on the developing nerves (i.e., alcohol is also a known teratogen) (Walsh & McLeod, 1970; Juntunen *et al*., 1978; Bosch *et al*., 1979; Pinazo-Duran *et al*., 1993; Rice & Barone, 2000; Sawada *et al*., 2002; Koike *et al*., 2003; Ertem *et al*., 2009; Chopra & Tiwari, 2012; Nguyen *et al*., 2012; Mellion *et al*., 2013; Kashkin *et al*., 2016; Igit & Colcimen, 2019; Tessitore *et al*., 2022). Alcohol (and its metabolites) has the unique ability to up–regulate oxidative stress, mitochondrial free radicals, and circulating cytokines which are responsible for the degeneration of sensory and motor nerves (Dina *et al*., 2000; Chopra & Tiwari, 2012; de La Monte & Kril, 2014). Alcohol also damages organic compounds such as DNA, proteins, and lipids which are essential for cell structure and functions (Tóth *et al*., 2014). The mechanism through which alcohol damages the myelin is not yet fully understood (Guerri & Pascual, 2016; Dudek *et al*., 2020) however it must be re-emphasized that the up–regulation of oxidative stress is one of the key processes through which alcohol exerts its neurodegenerative effects by triggering cellular apoptosis through events such as changes to organic mitochondrial proteins, lipids and DNA which ultimately results in a significant loss of neurons or cause nerve dysfunction (e.g., demyelination) (Goodlett & Horn, 2001; Wu & Cederbaum, 2003; Pascual *et al*., 2007; Brocardo *et al*., 2011; Chopra & Tiwari, 2012; Ehlers *et al*., 2013; de La Monte & Kril, 2014; Bhat et al., 2015). From the present study, alcohol significantly damaged the sciatic nerve in both sexes which means that alcohol effects on both sexes were similar.

In addition, the patterns of distribution of the g–ratio also confirms the damaging effects of alcohol. The distribution of axons with a g–ratio of >0.7 (i.e., Group III axons) was lower in the non–treatment or Simvastatin-only group than in the alcohol-only group for both sexes. The g–ratio is an important factor that is related to the degree of myelination which is essential for preserving, maintaining, and sustaining electrical signals within the axon. Thus, the g–ratio and myelination play a major role in the function and integrity of the axons (Marca *et al*., 2011; Cercignani *et al*., 2017). A high g–ratio indicates less myelination and neurodegeneration which may be caused by aging, the effect of drugs, or neurological disorders (Jeronimo *et al*., 2005; Fancy *et al*., 2009; La Marca *et al*., 2011; Makinodan *et al*., 2012; Xie *et al*., 2014). In comparison to the distribution of axons in Group I (i.e., <0.6), it seems that the majority of the axons in the non–treatment or Simvastatin-only group were composed of axons with hypermyelination, and which is an indication of axonal regeneration or newly developed axons (Fancy *et al*., 2009; La Marca *et al*., 2011; Makinodan *et al*., 2012; Xie *et al*., 2014). On the other hand, the distribution of axons in Group II which indicates axons with an ideal g–ratio was slightly more abundant in the non–treatment or Simvastatin only than in the alcohol, especially for the male mice. These findings further affirm the damaging effects of alcohol on the structure of the sciatic nerve, and which may negatively impact the function of the nerve and may explain the reason for the sensorimotor deficits that are often seen in alcoholics.

The neuroprotective properties of Simvastatin at two different concentrations against alcohol–induced peripheral neuropathy were explored in the present study. The findings revealed that both concentrations of Simvastatin were effective against the effect of alcohol on axonal density in the females but not in the males. In addition, myelin thickness was also improved by both concentrations of Simvastatin in the females but only the low concentration of Simvastatin was effective in the males. Due to a lack of information in the literature, the present study could not make direct comparisons however the neuroprotective effect of Simvastatin had been reported in a study by Namazi *et al*. (2019). The study reported that 8 mg/Kg Simvastatin administered to female rats for one month improved sciatic nerve regeneration following Wallerian degeneration as a result of a surgically induced nerve injury. In another non–alcohol–related study, 7.5 and 15 mg/Kg (but not 30 mg/Kg) Simvastatin administered for a period of 10 days reversed Vincristine–induced neuropathic pain in adult male rats due to the anti-inflammatory properties of Simvastatin (Bhalla et al., 2015). Likewise, in another study that used Atorvastatin (another type of Statin), it was reported that the oral administration of 5mg/Kg Atorvastatin was effective for promoting axonal regeneration, which improved axonal density following a crush–induced injury on the sciatic nerve of male rats. The study attributed the regenerative effects of

Atorvastatin to the ability of Atorvastatin to increase the expression of neurotrophic factors which is necessary for the proliferation of Schwann cells and axons (Pan et al., 2010). However, a 14–day treatment with 5 mg Atorvastatin did not significantly improve the population of myelinated regenerating axons following complete transection of the sciatic nerve of adult female Sprague-Dawley rats (Cloutier et al., 2013).

It is thus suggested that the improved axonal density and myelination found in the present study could be due to the anti-oxidative and anti-inflammatory properties of Simvastatin as these factors have been demonstrated to be vital to nerve structure and functions (Esposito *et al*., 2012; Zhang *et al*., 2019; Mansouri *et al*., 2022). Although these factors were not evaluated in the present study, it is assumed that the anti-oxidative and anti-inflammatory properties of Simvastatin inhibited or suppressed the ability of alcohol (or its metabolites) to up–regulate oxidative stress which is a known pathway through which alcohol exerts its neurogenerative effects (Nasef et al., 2021). In addition, the regenerative capability of Simvastatin has been reported on the sciatic nerves (Xavier et al., 2012) and the brain (Ma et al., 2021) and Atorvastatin on the sciatic nerve (Cloutier *et al*., 2013; Bolandghamat *et al*., 2022). It is believed that key processes such as axonal sprouting that occur during peripheral nerve regeneration contribute to the improvement of axonal densities (Jianping et al., 2012).

Another important observation in the present study was that the low concentration (5 mg) of Simvastatin was generally more effective against alcohol–induced peripheral neuropathy than the higher concentration. The observed trend is similar to the other reports in the literature which reported Simvastatin concentration of ≤8 mg to be effective (e.g., Shi *et al*., 2011; Pathak *et al*., 2013;Bhalla *et al*., 2015; Corso *et al*., 2018; Namazi *et al*., 2019), however, it should be noted that Bhalla *et al*, (2015), Corso *et al*., (2018) and Pathak *et al*., (2013) also reported that ≥10mg Simvastatin was also effective. In addition, the effect of Simvastatin on alcohol–induced peripheral neuropathy seems to be sex–specific which may highlight a possible influence of an underlying biological factor with respect to axonal density and myelination. Sex plays an important role in the pharmacokinetics and clinical efficacy of drugs during treatment (Meibohm *et al*., 2002). It is also true that human females are 50 – 75% more likely to experience drug–related reactions (Rademaker, 2001), and drug metabolism is mostly influenced by sex (Waxman & Holloway, 2009). Simvastatin is first metabolised in the liver by the CYP3A4 enzyme (a subset of the CYP40 superfamily of enzymes) before being excreted by the kidneys (Ward et al., 2019). The CYP3A4 enzyme mRNA and protein expression and their activities have been reported to be higher in women than in men (Rademaker, 2001;

Wolbold *et al*., 2003). Thus Simvastatin is known to metabolise faster in females than in males in humans (Vree et al., 2003). Likewise, Atorvastatin has a faster uptake and clearance in female rats than in male rats – an indication of higher efficacy (Clemente et al., 2021). For these reasons, the present study is of the opinion that the better improvement of Simvastatin in females could be attributed to the faster metabolism and higher efficacy of Simvastatin in females than in males. Further studies are needed to better understand the underlying reasons for the differences between the two sexes.

**Simvastatin significantly prevented barrel–specific alcohol damage in the somatosensory barrel cortex**

From the results of the present study, the administration of alcohol did not significantly reduce the sizes of the barrels or their septal portions for both sexes. These findings align with the similarities in the barrel arrangement of the PMBSF across the experimental groups as they appeared normal and typical of the normal arrangements found in rodents (Margret *et al*., 2005; Powrozek & Zhou, 2005; Margret *et al*., 2006; Chappell *et al*., 2007; Olateju *et al*., 2019). Unfortunately, there is a lack of information in the literature to directly compare the findings of this study. Interestingly, other studies that evaluated the effect of alcohol during fetal development also reported no statistically significant reduction in the barrel size of the PMBSF or its septal portion of rodents following prenatal alcohol exposure (Chappell et al., 2007; Olateju et al., 2019). In contrary, other studies reported the neurodegenerative effect of alcohol on the average total area of the PMBSF barrels, the average size of individual PMBSF barrels, the area of PMBSF barrel enclosure, and the septal area of the PMBSF following prenatal exposure in juveniles (6weeks) and adult (7 months) prenatal alcohol exposure (PAE) rats (Chappell et al., 2007), neonatal (Margret et al., 2006) PAE rats (Margret *et al*., 2005; Margret *et al*., 2006) and (PND 7) PAE mice (Powrozek & Zhou, 2005) or postnatal alcohol exposure in neonatal (PND 10) rats (Oladehin et al., 2007).

Differences in methodologies (e.g., prenatal vs postnatal; fetus vs adolescent vs adult; alcohol concentration and mode of alcohol administration) between the present study and other studies may have contributed to the differences in the outcomes of the effect of alcohol. One important factor is the growth period during which alcohol is administered as alcohol is more detrimental to the developing fetus's brain (Chen *et al*., 2003; Abbott *et al*., 2016) such as in the case of Fetal alcohol spectrum disorder. Likewise, the alcohol concentration is another important factor in the methodology which may have impacted the outcomes.

Thalamocortical connectivity and septal circuitry are imperative for the proper development of the somatosensory cortex (Toulmin et al., 2021) and these connections develop extensively during late fetal life (Toulmin et al., 2021) and they continue to mature and strengthen with age (Fair et al., 2010). Thus, it could be that the somatosensory cortex is less vulnerable to the effect of alcohol in early–to–late adolescence as is the case in the present study compared to during fetal life where thalamocortical connectivity and functionality are severely and directly affected by alcohol (Alkonyi et al., 2011). This is an indication that the general morphology of the PMBSF, specifically the barrel organization and topography, remains intact and preserved in adolescence despite the administration of alcohol (or Simvastatin – see discussion below) (Petersen, 2007; Olateju *et al*., 2019). Alcohol, especially prenatal exposure, has been shown to affect the terminal ends, specifically and directly, of thalamocortical axons thus severely affecting the septal circuitry of the somatosensory cortex (Minciacchi *et al*., 1993; Granato *et al*., 1995), and in turn, impairing the normal brain function i.e., sensory–motor functions. This assertion is supported by Lotfullina & Khazipov (2018) that concluded that the inhibitory effect of alcohol on the neural activity of the somatosensory cortex is most severe in neonates than in adolescents or adults.

In addition, both concentrations of Simvastatin did not have any effect on the PMBSF barrels in both sex. Likewise, in the presence of a 'silent and insignificant effect of alcohol,' the barrel sizes were similar across the experimental groups. However, the significant improvement of the area of the septal portion of the PMBSF of the females by the administration of 5 mg Simvastatin in the presence of alcohol is an interesting observation despite observing no difference between the alcohol and the non-treatment groups. Interestingly, this further shows that the thalamocortical fibres in the septal portion of the PMBSF barrels are mostly impacted by drugs. In a study on another part of the brain, Jafari *et al*. (2021) reported that the administration of 10 or 20 mg/Kg Simvastatin significantly reduced the impact of alcohol–induced cell death of hippocampal neurons. In the same study, behavioural and biochemical analyses revealed that Simvastatin significantly improved the functionality of the hippocampus following postnatal alcohol exposure (5.25g/kg) to pups of Wistar rats. Nasef *et al*. (2021) also reported that a 55–day administration of 10 mg/Kg Simvastatin prevented alcohol–induced neurodegeneration in the brains of female Swiss Albino rats. Comparing the outcomes of the present study with that of Jafari *et al*. (2021) and Nasef *et al*. (2021), it seems that the effect of Simvastatin may be specific to certain brain areas e.g., the hippocampus, or a specific neural structure e.g., thalamocortical axons/fibres.

On the other hand, when a barrel–to–barrel comparisons were performed, alcohol significantly affected specific PMBSF barrels in specific rows and arcs. With respect to sex, only PMBSF barrels A2 and A3 of the female alcohol-only group were significantly larger than the other experimental groups while PMBSF barrels D5, and E2 were significantly affected by alcohol in the males. In addition, PMBSF barrels in row A and Arc 1 in females and row E and straddler barrels were affected by alcohol in the males. These findings are consistent with other reports that evaluated the effects of prenatal effects of alcohol on the PMBSF barrels (Margret *et al*., 2005; Chappell *et al*., 2007; Olateju *et al*., 2019). For example, Olateju *et al*. (2019) reported that barrels in rows D and E were significantly affected by prenatal alcohol while all the barrels in the rows and arcs (except row A and straddler arc) were affected by prenatal alcohol in the study by Chappell *et al*. (2007).

It is however surprising that the effect of alcohol was either significantly increased or decreased in some specific barrels. Unfortunately, the present study could not explain the reasons for the varying degrees of alcohol effect in these specific PMBSF barrels, however, several reports have demonstrated that alcohol induced neurodegeneration in the brain (Morris et al., 2010; Sullivan et al., 2010) while other studies have reported an increase in the regeneration of neurons in order to compensate for the significant loss of neurons caused by alcohol (Miller, 1995; Bartsch *et al*., 2007) thus helping the brain to recover from the alcohol insult (Nawarawong et al., 2021). For example, Åberg et al. (2005) reported an increase in hippocampal neurogenesis following a 10–week administration of alcohol to female adult C57BL/6J mice. Compensatory mechanism is often reported in hippocampal neurogenesis (Miller, 1995; Nixon & Crews, 2004; Nixon *et al*., 2008; He *et al*., 2009; Hansson et *al*., 2010; Somkuwar *et al*., 2016; Hayes *et al*., 2018; Nawarawong *et al*., 2021), however, it is a phenomenon that could be triggered in other regions of the brain (Carlen et al., 1984; Durazzo et al., 2017).

The brain possesses a modest degree of postnatal neurogenetic capability (Altman & Das, 1965, 1966; Eriksson *et al*., 1998), however, this ability decreases with age (He & Crews, 2007) where it begins to plateau at mid-adolescence (Altman & Das, 1965, 1966) and it is hardly detectable by adulthood (Kuhn et al., 1996). The human brain can repair itself through neurogenesis following trauma/injury (Zheng et al., 2013) or pathology e.g., Huntington's disease (Curtis et al., 2003) and stroke (Jin et al., 2006), and even in the brains of rodent models of Alzheimer's disease (Jin et al., 2004) and experimental demyelination (Calz et al., 1998). Thus, the present study believes that the increased size of some PMBSF barrels was due to the triggering of a

compensatory mechanism to recover from the damaging effects of alcohol. At the same time, Simvastatin may have moderated or regulated the cellular activities in these specific barrels evidenced by some of the similarities between the control (i.e., NT or SIM) and the treatment groups (i.e., ALC+SIM5 or ALC+SIM15) in the specific PMBSF barrels being discussed. The ability of Simvastatin to regulate cellular activities and functions has been well-documented in other organs (Wu *et al*., 2009; Satirapoj *et al*.,2015; Porter & Turner, 2011; da Silva Pereira *et al*., 2022) and it is suggested that such would have come to play in the PMBSF barrels in the present study.

In conclusion, the present study affirmed the damaging effects of alcohol on the sciatic nerve and also on some specific barrels of the somatosensory cortex of adolescent mice. This may be the reason for the sensory–motor delays or impairment often seen in alcoholics as information from sensory nerves are relayed to the somatosensory cortex for processing and interpretation. Thereafter, actions are then executed by the motor cortex. It is probable, any damage to the structures in the sensory–motor pathway may severely impair, or delay motor functions as seen in alcoholics with alcohol–related peripheral neuropathy or impaired cerebral functions. This problem continues to be a health issue and a financial burden to the Government of South Africa. Thus, there is a need to reduce the impact of this problem in order to reduce the prevalence of adolescent alcoholism and the health and social problems associated with it. Thus Simvastatin, a cost–effective and an FDA–approved drug, has the benefits of preventing the onset of alcohol–related peripheral neuropathy and the impairment of somatosensory functions. Although the effectiveness of Simvastatin seems to be dose–dependent and sex–specific, further studies are needed to better understand the underlying reasons for these differences. It is expected that the outcomes of this study will further highlight the negative impacts of alcohol misuse and will increase awareness of the versatility of Simvastatin not only as a drug for the lowering of blood cholesterol but a drug that can be used for the treatment or prevention of other health problems such as peripheral neuropathy or cerebral–related problems. The advantage of using Simvastatin is that it is already approved for human use and thus its adoption (if additional studies have further proven its effectiveness) for treating other health conditions will be much faster. This is also expected to be advantageous to lowering the prevalence of alcohol–related problems and the cost to the Government of South Africa.

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# APPENDICIES

## APPENDIX A: ETHICS CERTIFICATE

ANIMALS RESEARCH ETHICS COMMITTEE (AREC)

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

**STRICTLY CONFIDENTIAL**

CLEARANCE CERTIFICATE NUMBER: 2019/11/63/C

**APPLICANT: Dr O Olateju**
**School: School of Anatomical Sciences; Department: N/A;**
**Location: CAS**

**PROJECT TITLE: Does simvastatin have neuroprotective effects against Alcohol-induced brain damage in C57BL/6J**

**Category: C; Species and Numbers involved:** *150X 3 Weeks old (9-15g) male Mus musculus C57BL/6J and 150X 3 Weeks old (9-15g) female Mus musculus C57BL/6J*

Approval is hereby given for the use of animals for the research project named above and described in the application reviewed by a quorate meeting of the AREC held on 1 Oct 2019. This approval remains valid until 20 Jan 2022.

All material changes to the approved research must be reported to the AREC before they are implemented. Failure to do so will invalidate this clearance certificate.

An annual progress report must be provided to the AREC.

The use of these animals is subject to AREC guidelines on the use and care of laboratory animals, is limited to the procedures described in the application and is subject to additional conditions listed below:

I, the Chair of the AREC (or my designated representative) am satisfied that the proposed research is ethical as judged by local law, international standards and University policy.

Signed: _____ Date: 28th FEBRUARY 2020
(Chairperson of the AREC)

I am satisfied that the persons listed in this application are competent to perform the procedures described in the application, in the context of Section 23 (1) (c) of the veterinary and Para-veterinary Professions Act (19 of 1982).

Signed: _____ Date: 28 February 2020
(Registered Veterinarian)

CC: Student supervisor: N/A N/A
Director Central Animals Service: Dr Kim Jardine

APPENDIX B: TURNITIN REPORT

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