HYPERGLYCINEMIA IN CAPTIVE-BRED VERVET MONKEYS WITH CATARACTS: GENETIC DYNAMICS AND ASSOCIATIONS

By

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Vervet monkey

ABSTRACT

Hyperglycinemia in captive-bred vervet monkeys with cataracts: genetic dynamics and associations

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PhD thesis, Department of Medical Biosciences, University of the Western Cape

A small percentage (8%) of the captive-bred vervet monkeys (*Chlorocebus aethiops*) maintained at Primate Unit and Delft Animal Centre (PUDAC) of the South African Medical Research Council (SAMRC) were found to have high levels of glycine in their plasma (457-795 µmol/L) and cerebrospinal fluid (CSF) (7.5-12.7 µmol/L). Additionally, these hyperglycinemic monkeys developed cataracts, a condition which has been previously characterized and reported in this specific colony of captive-bred vervet monkeys. This type of association has never been reported in literature before, therefore, this study will be the first of its kind to be investigated in non-human primates (NHPs). Nonketotic hyperglycinemia (NKH), also known as glycine encephalopathy, is well characterised in humans. The symptoms are exclusively neurological in nature, and clinically patients are diagnosed with abnormally high glycine levels in plasma (normal <350 µmol/L) and CSF (normal range 0-8). This neurological disorder is transmitted in an autosomal recessive form and is mainly instigated by a defective glycine cleavage system (GCS). In contrast to GCS, glycine transporter (GlyT1) which regulates glycine concentration at synapses and valproate administration have been associated with NKH.

In this study, it was hypothesized that a correlation exists between hyperglycinemia and cataract in vervet monkeys. Since there is a close genetic relatedness between humans and NHPs, underlying genetic factors associated with cataract and hyperglycinemia in vervet

monkeys are similar to those found in humans. Hence, genes that are implicated in cataract and NKH in humans were considered candidate genes in vervet monkeys.

Two approaches were followed: (1) The animal intervention approach with valproate, sodium benzoate and dextromethorphan was conducted to compare the effectiveness of the current NKH treatment; (2) Molecular aspects of NKH were investigated using genotyping and gene expression techniques for valproate glucuronidation (*UGT1A6* and *UGT1A9*), GlyT1 (*SLC6A9*), GCS (*AMT* and *GLDC*) and cataract (*CRYAA* and *GCNT2*) genes.

Based on the findings from the animal intervention study, valproate induction in phase one elevated alkaline phosphatase, phosphate and platelet count. In phase two, the effect of valproate on the aforementioned biochemical parameters were reversed by sodium benzoate and dextromethorphan. The treatment was more effective in reducing glycine levels in plasma and CSF of the spontaneous group. Furthermore, the genotyping results for *UGT1A6* revealed four missense variants, three silent variants in *UGT1A9* and one silent variants in *SLC6A9*, and these sequence changes were not identified in the control group. Therefore, it is possible that these sequence variants played a role in valproate metabolism during the intervention study. A similar observation was made between mutated spontaneous individuals compared to the controls, and the results showed that both *AMT* and *SLC6A9* genes were down-regulated in the spontaneous group. Therefore, *AMT* and *SLC6A9* gene expression confirmed that there is a link between cataract formation and hyperglycinemia.

The findings of the study conclusively suggest that a combination of drug therapy of sodium benzoate and dextromethorphan can be considered as treatment for normalizing glycine levels

in plasma and CSF. Additionally, GCS and GlyT1 sequence variants may be responsible for the spontaneous hyperglycinemia in captive-bred vervet monkeys.

DECLARATION

I declare that **"*Hyperglycinemia in captive-bred vervet monkeys with cataracts: genetic dynamics and associations*"** is my own work and has not been submitted for any degree or examination in any other University. All the sources that I have used or quoted have been indicated and acknowledged by means of complete references.

Full name: Zandisiwe Emilia Magwebu **Date:** 06 November 2017

Signed: 

LIST OF ABBREVIATIONS

ActB	Beta actin
AED	Antiepileptic drug
ALP	Alkaline phosphatase
ALT	Alanine Aminotransferase
AMT	Aminomethyltransferase
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
beats/min	beats/minute
BRT	Blood RNA Tube
bs	Brain stem
Ca	Calcium
cb	Cerebellum
cDNA	Complementary deoxyribonucleic acid
Cl	Chloride
CK	Creatine Kinase
CNS	Central nervous system
CoA	Coenzyme A
CO_2	Carbon dioxide
CSF	Cerebrospinal fluid
DLD/GCSL	Dihydrolipoyl dehydrogenase
DNA	Deoxyribonucleic acid
ECRA	Ethics Committee for Research on Animals
EDTA	Ethylene-Diamine-Tetra-Acetic acid
et al	*et alli*: and others

ey	Eye
fb	Forebrain
fl	femtolitre
g	Gram
GABA	Gamma-aminobutyric acid
GCNT2	Glucosaminyl (N-acetyl) transferase 2
GCS	Glycine cleavage system
GCSH	Glycine cleavage system H
GGT	Gamma Glutamyl Transferase
git	Gastrointestinal tract
GLDC	Glycine dehydrogenase
g/dL	gram/decilitre
g/L	gram/litre
GLYAT	Glycine N-acyltransferase
GLYT1	Glycine transporter 1
GLYT2	Glycine transporter 2
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Hb	Haemoglobin
HCT	Haematocrit
HDL-C	High-density lipoprotein cholesterol
H-protein	Lipoic acid
K	Potassium
Kg	Kilogram
L	Liter
LDH	Lactate Dehydrogenase

LDL-C	Low-density lipoprotein cholesterol
li	Liver
L-protein	Lipoamide
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCM	Methylmalonyl-CoA mutase
MCV	Mean corpuscular volume
Mg	Magnesium
MMA	Methylmalonic acidemia
mg/kg	milligram/kilogram
min	Minutes
ml	Milliliter
mm	Millimeter
mM	Millimolar
mm/Hg	millimetre Mercury
mmol/L	millimol/litre
mRNA	Messenger ribonucleic acid
Na	Sodium
NCBI	National Center for Biotechnology Information
ng	Nanogram
NH_3	Ammonia
NKH	Nonketotic hyperglycinemia
nm	Nanometer
NMDA	N-methyl-D-aspartate
ob	Olfactory bulb

OD	Optical density
pa	Pancreas
PA	Propionic acidemia
PCC	Propionly–CoA carboxylase
PCR	Polymerase chain reaction
pg	Picogram
PGK1	Phosphoglycerate kinase 1
PHS	Public Health Service
P- protein	Pyridoxine
PUDAC	Primate Unit and Delft Animal Centre
RBCs	Red blood cells
RDW	Red blood cell distribution
RIN	RNA integrity number
RNA	Ribonucleic acid
qRT-PCR	Quantitative Real-Time polymerase chain reaction
*SA*MRC	*South African* Medical Research Council
SANS	South African National Standard
sc	Spinal cord
SHMT	Serine hydroxymethyl transferase
SST	Serum Separator Tube
Taq	*Thermus aquaticus*
TBE	Tris Borate EDTA
THF	Tetrahydrofolate
Tm	Melting temperature
T- protein	Tetrahydrofolate

µg	Microgram
UGT	Uridine 5`diphosphoglucuronosyl-transferase
µl	Microliter
u/L	Units/litre
µM	Micromolar
umol/L	Micromol/litre
UV	Ultraviolet
V	Volt
WBC	White blood cells
%	Percentage
°C	degrees Celsius

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CHAPTER ONE:

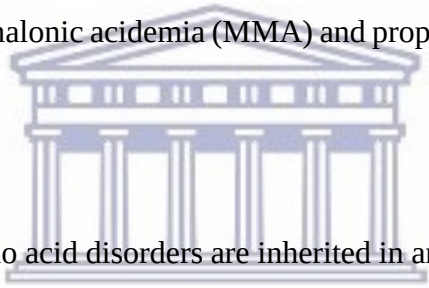
General background

1.1 GENERAL INTRODUCTION

High glycine levels were observed in the plasma (457-795 µmol/L, normal <350 µmol/L) (Applegarth and Toone, 2001) and CSF (7.5-12.7 µmol/L, normal range 3-8) (Dinopoulos et al., 2005, Ekici et al., 2011) of a small percentage (8%) of captive-bred vervet monkeys maintained at PUDAC, *SA*MRC. Human patients with such high glycine levels (CSF/Plasma ratio >0.08), are diagnosed with nonketotic hyperglycinemia also known as glycine encephalopathy (Pai et al., 2015). The disorder is a rare autosomal recessive inborn error of amino acid in neonates, juveniles and adults (Lu et al., 1999). It is characterised by accumulation of large quantities of glycine in all body tissues (CSF, plasma and urine) including the brain (Applegarth and Toone, 2006, Hennermann et al., 2012, Hennermann, 2006). The underlying defect is in the glycine cleavage system (GCS), which is an enzyme responsible for glycine oxidative decarboxylation in the mitochondria (Hamosh and Johnston, 2001, Dinopoulos et al., 2005, Tada and Kure, 1993, Tan et al., 2007, Kikuchi et al., 2008).

The same monkeys also suffered from total cataract phenotype, which is a pathological condition that results in clouding of the normally clear crystalline lens of the eye (Hejtmancik, 2008, Francis et al., 1999, Song et al., 2009). Though cataracts have been documented for a variety of primate species such as vervet, rhesus and cynomolgus monkeys, NKH has not been studied in NHPs, and hence the association between the two disorders needs to be investigated. Although NKH is mainly caused by defective GCS, it is also caused by a defective glycine transporter 1 (GlyT1) which removes glycine from inhibitory glycinergic synapses in

brainstem, spinal cord and hippocampus (Zafra et al., 1995, Legendre, 2001). Failure of glycine GlyT1 to prevent saturation of glycine-binding site (Betz et al., 2006) results in high glycine levels in the CNS, hence high glycine are found in CSF (Viljoen et al., 2012). In knockout mice, removal of GlyT1 resulted in symptoms such as lethargy, hypotonia and hyporesponsitivity which are similar to the symptoms found in NKH patients (Gomeza et al., 2003). Furthermore, administration of therapeutic compound such as valproate also triggers drug-induced hyperglycinemia. Subsequently, valproate causes high glycine levels (Morrison et al., 2006), decreased GCS activity and increased seizure frequency (Dhamija et al., 2011, Ekici et al., 2011, Tsuyusaki et al., 2012, Verissimo et al., 2013, Hamosh et al., 1992). However, it should be noted that hyperglycinemia is also an indication of other inborn errors of metabolism such as methylmalonic acidemia (MMA) and propionic acidemia (PA) (Kruszka et al., 2014).



The NKH, MMA and PA amino acid disorders are inherited in an autosomal recessive manner and occur due to deficiency in enzyme activity. In humans, these disorders have similar clinical features such as vomiting, poor feeding, seizures and lethargy which can be distinguished by performing urine organic analysis (Baumgartner et al., 2014). In contrast to NKH, MMA is characterized by excessive excretion of methylmalonic acid in plasma and urine (Saudubray et al., 2011) due to defective methylmalonyl-CoA mutase (MCM), an enzyme responsible for converting methylmalonyl-CoA into succinyl-CoA (Kasahara et al., 2006). Conversely, PA is characterized by accumulation of propionic acid which is found in high levels in the urine resulting in ketoacidosis (Sebens, 2011). In general, the propionly–CoA carboxylase (PCC) enzyme is responsible for the metabolism of essential amino acid such as isoleucine, valine, threonine and methionine (Sebens, 2011, Guenzel et al., 2013, Baumgartner et al., 2014). During the metabolism, propionyl-CoA is converted to methylmalonyl-CoA by the PCC

enzyme (Baumgartner et al., 2014, Pena et al., 2012). Similarly, both MMA and PA disorders are characterized by the presence of metabolites such as methylcitrate and 3 hydroxypropionic acid (Baumgartner et al., 2014) in their body fluids (CSF, plasma and urine). Therefore, NKH can be distinguished from MMA and PA by high CSF/plasma ratio (≥0.08), absence of ketones in urine and normal levels of organic amino acid (e.g propionic acid and methylmalonic acid) (Sehgal and Ramji, 1998, Applegarth and Toone, 2006, Baumgartner et al., 2014, Applegarth and Toone, 2001).

Currently, there is no specific cure for NKH, a combination of sodium benzoate and dextromethorphan can be utilised as alternative treatment to normalise glycine levels (CSF: plasma ratio <0.02) (Arnold et al., 1997, Hussain et al., 2013, Hoover-Fong et al., 2004). It is worth mentioning that there are conflicting findings about the effectiveness of the treatment in severe cases of NKH (Neuberger et al., 2000, Randak et al., 2000, Van Hove et al., 2005). Lu et al. (1999) have reported the treatment to have a neurological improvement effect on classical NKH new-borns, however, reduction of glycine levels in their CFS was not observed. On the contrary, Hamosh et al. (1998) reported an improved arousal, decreased or eliminated seizures, and some developmental progress in four treated NKH infants. Based on Hamosh's findings, it is evident that the treatment is effective in some cases. Nevertheless, sodium benzoate (200-750mg/kg/day) decreases the concentration of glycine in blood (<350µmol/L), urine and CSF (Van Hove et al., 2005, Neuberger et al., 2000). Conversely, dextromethorphan is used as an antagonist for N-methyl-D-aspartate (NMDA) (Randak et al., 2000, Hamosh et al., 1990) and to minimize seizures in NKH patients (Sehgal and Ramji, 1998, Hamosh et al., 1992).

This chapter will mainly summarize information about NKH, glycine's role at NMDA receptors, glycine metabolism (GCS pathway), molecular studies on GCS components (Table

1.2) and glycine transporter 1 (*SLC6A9*). Additionally, the metabolism of the treatment components of valproate, sodium benzoate and dextromethorphan will also be covered in the literature review. This chapter will end by providing an overview of the dissertation.

1.2 NONKETOTIC HYPERGLYCINEMIA

In humans, there are two different forms of NKH observed; classical (neonatal) and atypical form (infantile, late onset and transient) (Kure et al., 2006). Clinical presentation of NKH differs for each form (Roy et al., 2003) and is categorized according to age of onset, clinical presentation and the severity of the disease (Table 1.1) (Bhamkar and Colaco, 2007, Ramirez et al., 2012). Patients with classical form of NKH show severe neurological symptoms such as apnea, hypotonia, lethargy, myoclonic jerks, seizures and respiratory distress in the first few hours to days (Hoover-Fong et al., 2004, Boneh et al., 2008, Hennermann et al., 2012, Roy et al., 2003). For atypical NKH (transient, infantile and late onset), the biochemical and clinical symptoms of transient NKH are similar to classical form, however, the transient form can be partially or completely resolved within days or months without treatment (Lu et al., 1999). Children with this transient form have rarely been reported (Hennermann, 2006).Whereas patients with infantile form are presented after six months with hypotonia, progressive spasticity as result of intractable seizures, developmental delays, severe psychomotor symptoms and mental retardation (Hennermann, 2006, Ramirez et al., 2012). The late onset of atypical NKH is presented in children from two years with cognitive decline and behavioural problems (Hennermann, 2006), however, patients tend to have a normal quality lifespan without the occurrence of seizures (Dinopoulos et al., 2005).

Table 1.1: Different forms of NKH based on glycine levels in CSF and plasma

Laboratory data	Normal levels	Classic NKH	Late onset NKH	Transient NKH
Glycine in plasma (µmol/L)	<450	420-4090	354-961	240-2285
Glycine in CSF (µmol/L)	3-10*	40-1440	7.4-68	16-463
CSF-plasma ratio	0.012-0.04	0.09-0.25	0.02-0.07	0.04-0.88

Normal glycine levels differ between laboratories and depend on age of onset. Glycine levels for different NKH forms were adapted from (Hennermann, 2006). Glycine plasma levels were adapted from (Applegarth and Toone, 2001) and glycine levels in CSF were adapted from *(Ferraro and Hare, 1985) (Sellner et al., 2005, Dinopoulos et al., 2005).

1.2.1 Neuropathogenesis of nonketotic hyperglycinemia

Glycine plays a vital role in the CNS by acting as an inhibitory neurotransmitter in the brainstem, retina and spinal cord, and as an excitatory neurotransmitter at inhibitory glycinergic synapses (Kure et al., 1997, Eulenburg et al., 2005). The inhibitory role is responsible for respiratory distress symptoms, while convulsion is observed as a result of excitatory role on NMDA receptors (Sakata et al., 2001). Therefore, GCS regulates extracellular glycine concentration in the vicinity of NMDA receptors rather than inhibitory glycine receptors (Kojima-ishii et al., 2008). It has been reported in a mice model that high extracellular glycine is a determinant of susceptibility to seizures, ischemic vulnerability, locomotor activity and mood (Kojima-ishii et al., 2008). Consequently, the neurological damage observed in NKH patients is caused by overstimulation of NMDA receptors as a result of high glycine concentration (Kure et al., 1997). The NMDA receptors are excitatory glutamate receptors extensively distributed in the nervous system and are composed of two NR1 glycine binding subunits and two NR2 glutamate binding subunit (Figure 1.1) (de Koning et al., 2007). These receptors play a role in regulating signal transduction in multiple regions of the brain and receptor activation requires the binding of both glutamate and glycine (de Koning et al., 2007).

This pathway then permits for cationic influx (Ca^{2+} and Na^{+}) from the synaptic cleft into the cell (Lakhan et al., 2013).

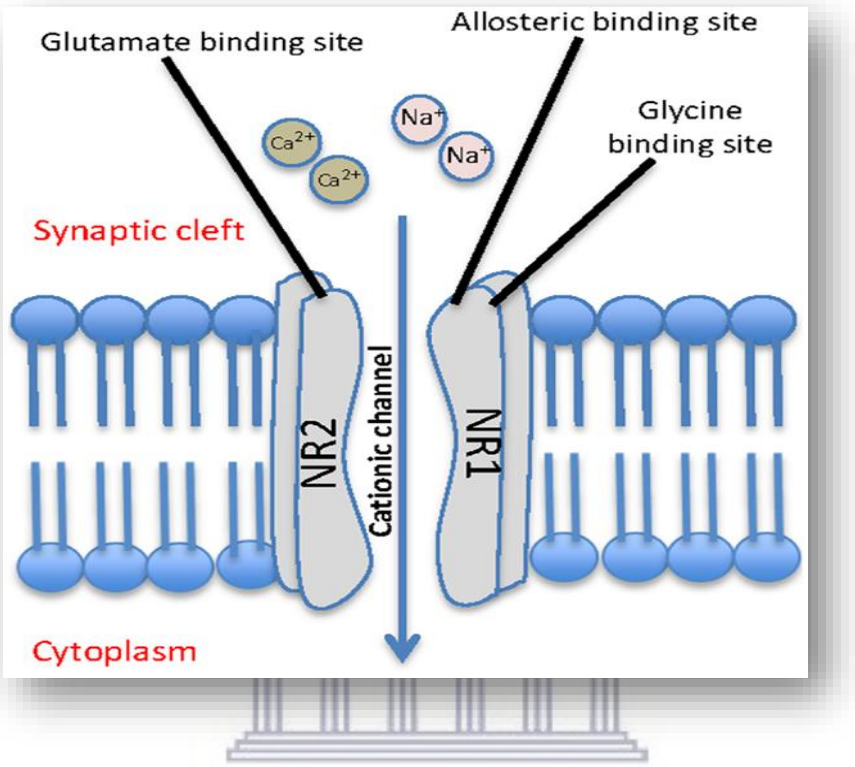


Figure 1.1. Schematic representation of a typical NMDA receptor. The NMDA contains two glycine binding NR1 subunits and two glutamate binding NR2 subunits (Lakhan et al., 2013).

1.2.2 Glycine metabolic pathways

Glycine is actively involved in the synthesis of many specialised products including purines, heme and creatine. It is required for the synthesis of serine and glyoxylate which occurs in a reversible manner. Glycine is eliminated in urine as hippurate from the circulatory system through three metabolic pathways (Figure 1.2). Thus far, GCS and serine hydroxymethlytransferase (SHMT) serve as the most important enzymes for glycine metabolism. The mitochondrial coupling of these two enzymes results in the conversion of glycine and serine (Mouillon et al., 1999, Lamers et al., 2007, Douce et al., 2001). Glycinergic neurons in the mammalian retina synthesise their own glycine from serine though there is no

evidence that SHMT is expressed in the retina. Glycine synthesised in this way does not remain as glycine, it is converted into a wide variety of other molecules such as glutathione. Hence, SHMT has been regarded as unreliable marker for glycinergic neurons (Pourcho and Goebel, 1990). Firstly, the GCS plays an important role in maintaining normal glycine levels in CNS and defects in the complex can ultimately result in NKH development (Pearl et al., 2005, Lamers et al., 2007). The GCS is the most prominent pathway for serine and glycine catabolism in humans and various vertebrates such as mammals, birds, reptiles, amphibians and fishes (Kikuchi et al., 2008). The GCS pathway requires the co-enzyme NAD, lipoamide and tetrahydrofolic acid to synthesise glycine.

The second pathway involves the synthesis of glycine from serine by SHMT, an enzyme that is dependant of tetrahydrofolate (THF) (Lamers et al., 2007, Van Hove et al., 2005). In the neurons, SHMT converts L-serine to glycine while in the glia it converts glycine to L-serine together with GCS (Verleysdonk et al., 1999). Serine dehydrates converts serine to pyruvate, which serve as a precursor for glucose (Figure 1.2). The conversion of serine by SHMT was thought to be the major route of glycine catabolism, however, NKH patients tend to have normal levels of SHMT enzyme (Tada and Kure, 2005), thus making GCS the major pathway.

The last glycine metabolism pathway is the D-amino acid oxidase (DAAO) pathway which entails the conversion of glyoxylate into glycine in the presence of lactate dehydrogenase (LDH) (Wang et al., 2013, Salido et al., 2012). The glyoxylate can be further converted to oxalate which then enters the one carbon pool. Additionally, glyoxylate contributes to the synthesis of glycine in the glycinergic neurons (Pow and Hendrickson, 1999). In this study, the GCS complex was studied further since it is directly associated with NKH when its activity is

abolished. Therefore, the remaining two pathways, serine and glyoxylate will not be discussed further.

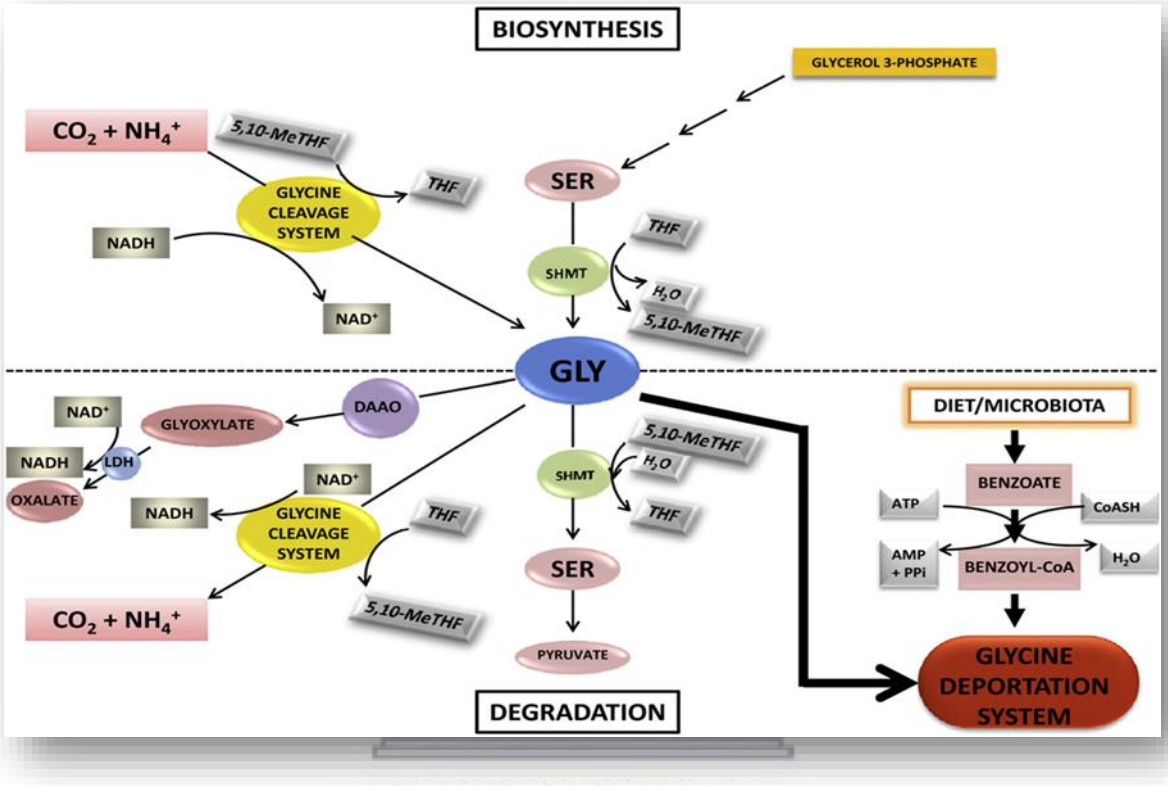


Figure 1.2: The overview of biosynthesis and degradation of glycine from the circulatory system, adopted from (Beyoglu and Idle, 2012).

1.2.2.1 Glycine cleavage system (GCS) pathway

Generally, GCS catalyses glycine in three steps and is completely reversible by catalysing the formation of glycine from 5, 10-CH2- Tetrahydrofolate (THF), ammonia (NH_3) and carbon dioxide (CO_2) (Figure 1.3) (Douce et al., 2001, Okamura-Ikeda et al., 2010). The first step of the reaction involves the decarboxylation catalysed by P-protein which requires H-protein to breakdown glycine. Whereby the remaining aminomethyl moiety is passed onto the distal sulphur of the lipoate cofactor of H-protein (Guilhaudis et al., 2000, Okamura-Ikeda et al., 2010). Therefore, an aminomethylated H-protein (Ham) is generated during the first part of the

reaction. Thereafter, the aminomethyl moiety is degraded by T-protein into NH_3 and 5, 10-CH_2- THF in the presence of THF (Okamura-Ikeda et al., 2010). During this step, the H-protein (Hred) has a reduced lipoate. In the third and final step, the reduced lipoate attached to H-protein (Hox) is reoxidized and this step is catalyzed by L-protein (Motokawa et al., 1995, Okamura-Ikeda et al., 2010). These GCS components are so important that any deficiency in GCS process can initiate NKH development.

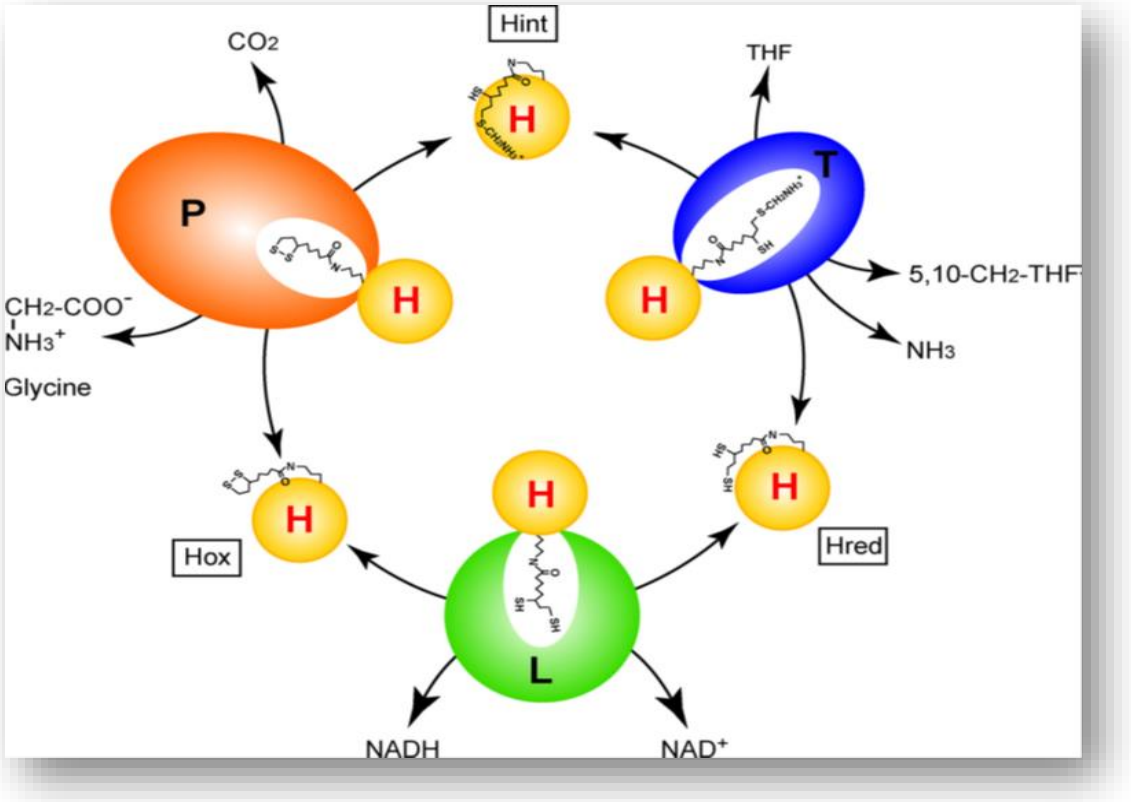


Figure 1.3: Outline of the reversible oxidative decarboxylation of glycine through GCS. Hox, Hint and Hred represent H-proteins bearing covalently attached lipoate (oxidized form), aminomethyllipoate, and dihydrolipoate (reduced form), respectively. Adopted from (Okamura-Ikeda et al., 2010).

1.2.2.2 Molecular involvement of glycine cleavage system (GCS) genes in NKH development

As previously shown (Figure 1.2), GCS mediates the degradation of glycine to form ammonia and carbon dioxide (Douce et al., 2001, Pearl et al., 2005, Lamers et al., 2007, Verissimo et al., 2013, Wang et al., 2013). The GCS system does not contribute much to the inhibitory role of glycine but modulate NMDA response by removing excessive synaptic glycine (Sakata et al., 2001, Tada and Kure, 2005). The genetic defects in GCS components are encoded by *GLDC*, *AMT* and *GCSH*, respectively (Table 1.2) (Applegarth and Toone, 2001, Kure et al., 2006). Various mutations in *AMT* and *GLDC* have been identified in neonatal, infantile and patients with late onset (Dinopoulos et al., 2005, Kure et al., 1998b, Kure et al., 1997, Kure et al., 2006, Nicolasjilwan et al., 2011, Swanson et al., 2015). It is believed that the *GLDC* gene accounts for 80% of mutations, 10-15% of mutations are associated with the *AMT* gene and rarely reported in *GCSH* (Toone et al., 2000, Applegarth and Toone, 2001, Azize et al., 2014, Hiraga et al., 1981). Nevertheless, mutations in any of these genes affect the overall activity of GCS (Kojima-ishii et al., 2008) and subsequently contribute to NKH development (Tada and Kure, 1993, Applegarth and Toone, 2001).

Table 1.2: GCS protein components associated with NKH

Gene name	Gene symbol	Protein	Chromosome position	No. of exons
Glycine dehydrogenase	GLDC	P	9p24	25
Aminomethyltransferase	AMT	T	3p21.31	9
Glycine cleavage system H-protein	GCSH	H	16q23.2	5

Adopted from (Kure et al., 1997, Tada and Kure, 2005)

1.2.2.2.1 Glycine dehydrogenase (GLDC) gene

Glycine dehydrogenase (*GLDC*) also known as P-protein consists of 25 coding exons (Figure 1.4) (Narisawa et al., 2012, Takayanagi et al., 2000) while there is 27 exons in rhesus monkeys (Chauke et al., 2016) and is highly expressed in the liver, kidney and brain (Kure et al., 1997). Due to the several exons involved, certain deletions in this gene can be overlooked during normal PCR and it is therefore recommended to use multiplex ligation-dependent probe amplification (MLPA) (Kanno et al., 2007). In humans, deficiencies in this gene are more responsible for neonatal and infantile forms of NKH, most of the reported mutations are reserved and specific for certain families or ethnic groups (Applegarth and Toone, 2001, Kanno et al., 2007, Applegarth and Toone, 2004). The same notion is reported for deletions (Sellner et al., 2005, Takayanagi et al., 2000, Kure et al., 1991, Azize et al., 2014). Furthermore, biochemical analysis of liver samples from NKH patients showed that 87% of the specimens had a reduced GCS activity in P-protein and the rest in T-protein (Kure et al., 1997). The same study confirmed that a three-base deletion was responsible for the reduced activity of the protein. A recent report indicated that defective *GLDC* in mice brought about NKH symptoms such as elevated plasma glycine and hydrocephalus (Pai et al., 2015). Therefore, *GLDC* might be responsible for NKH cases in both humans and rodents, thus far, no mutations have been reported in NHPs.

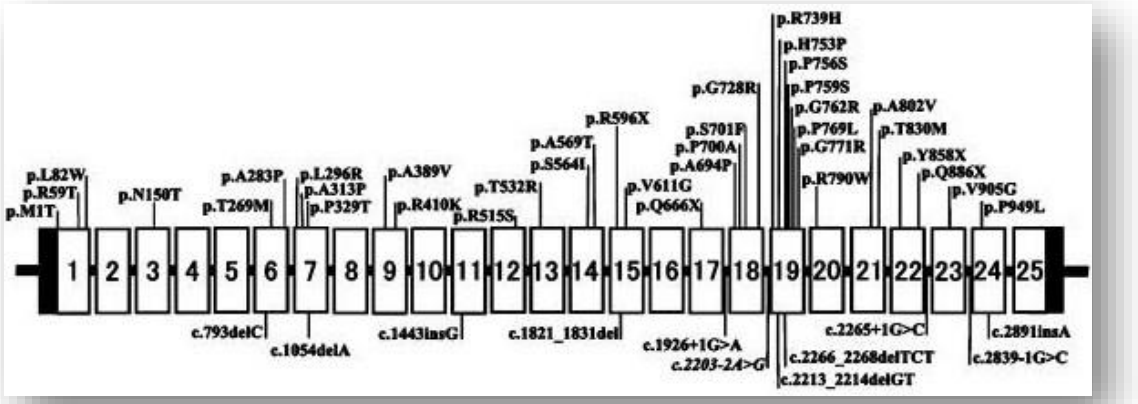


Figure 1.4: The *GLDC* exons showing NKH mutations that have been previously reported. The exons are indicated by open boxes, and noncoding regions are shaded. Missense and nonsense mutations are shown above the exon boxes, and deletions/insertions and mutations of splicing errors are indicated below the exon boxes (Kure et al., 2006).

1.2.2.2.2 Aminomethyltransferase (AMT) gene

The aminomethyltransferase (*AMT*) gene also known as T-protein, consists of nine exons (figure 1.5) (Nanao et al., 1994b) and is expressed in the liver, kidney, brain, spleen and heart (Kure et al., 1997). Mutations within this gene are reported to inactivate the human T-protein thus contributing to NKH development. The most common mutations include H42R (Kure et al., 1998a), R320H (Nanao et al., 1994a, Toone et al., 2000, Toone et al., 2001), and IVS7-1 G-A (Toone et al., 2001). The latter mutation is associated with the loss of human T-protein (Lee et al., 2004), while R320H interact with D276H mutation (Kure et al., 1998a).

It is believed that the interaction between R320H and D276H is necessary for proper functioning of T-protein, hence R320H is known to cause impaired T-protein activity (Lee et al., 2004). Additionally, *AMT* gene deficiencies have been associated with severe and atypical NKH forms. Although it has been speculated that mutations are more common in atypical cases (Hamosh and Johnston, 2001), it is challenging to follow up on these speculations since a large

amount of liver samples is not easily available and the confirmation will require a larger sample size (Toone et al., 2003).

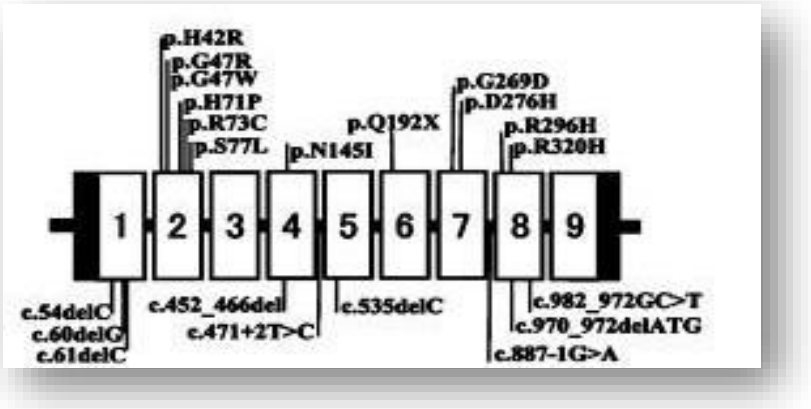


Figure 1.5: The *AMT* exons showing NKH mutations that have been previously reported. The exons are indicated by open boxes, and noncoding regions are shaded. Missense and nonsense mutations are shown above the exon boxes, and deletions/insertions and mutations of splicing errors are indicated below the exon boxes (Kure et al., 2006).

1.2.2.2.3 Glycine cleavage system H-protein (GCSH) gene

The human H-protein (GCSH) gene consists of five exons (Kure et al., 2001) and instigates less than 1% of NKH reported cases. The function of the gene is significant during the GCS reaction (Figure 1.2) by operating as a regulator and transporter to facilitate P-protein to initiate glycine degradation (Kikuchi et al., 2008). So far, only a defective H-protein (Hiraga et al., 1981, Trauner et al., 1981), one splicing mutation (Kure et al., 2002) and single polymorphism (Kure et al., 2001) have been reported in *GCSH*. In one of the reports (Trauner et al., 1981), a patients with defective H-protein was asymptomatic for atypical NKH and later became mentally retarded. There are no recent reports about the functionality of *GCSH* and its contribution to NKH development as it only accounts for 1% of the reported cases of NKH.

1.2.3 Glycine transporters involved in regulating glycine concentration at synapses

The glycine transporters are as important as GCS in maintaining glycine concentration at minimum levels to avoid saturation of NMDA receptors (Harvey and Yee, 2013). Upon the release of glycine into the synaptic cleft and binding to glycine receptors, glycine is removed from synaptic cleft by glycine transporter 1 (GlyT1) and GlyT2 (Eulenburg et al., 2005). Both transporters belong to the Na^+/Cl^--dependent transporters (Nelson, 1998) and transporters require the binding of two Na^+ and one Cl^- for transporting glycine. They share 50% of their amino acids but are expressed in different tissues and have different functions (Raiteri and Raiteri, 2010, Zafra and Giménez, 2008). The GlyT1 is expressed in glia cells of spinal cord and brain stem (Figure 1.6) and in close proximity to both inhibitory and excitatory synapses (Zafra et al., 1995). The GlyT1 function is to maintain glycine concentration at excitatory synapses containing NMDA receptors, thereby preventing saturation of glycine-binding site (Betz et al., 2006, Bergeron et al., 1998, Zafra and Giménez, 2008). Therefore, expression of GlyT1 correspond closely with NMDA expression (Smith et al., 1992).

Conversely, GlyT2 is expressed in neurons, spinal cord, brain stem and cerebellum and is responsible for re-uptake of glycine from the synaptic cleft (Betz et al., 2006, Eulenburg et al., 2005) (Eulenburg et al., 2005). Both transporters were thought to play a fundamental role in glycinergic inhibition due to overlapping expression patterns in spinal cord and brainstem (Gomeza et al., 2003). However, fluctuations in their activity seems to affect the efficacy of neurotransmission (Betz et al., 2006) with inadvertent to high glycine levels resulting in NKH development.

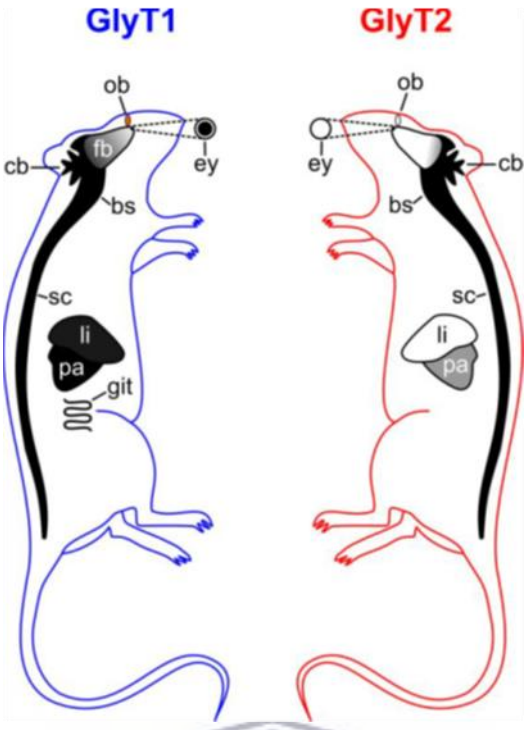


Figure 1.6: Distribution of GlyT1 and GlyT2. Schematic representation of the expression pattern of GlyT1 and GlyT2 in the mouse CNS. Both GlyTs are highly expressed in spinal cord (sc), brain stem (bs) and at lower levels in cerebellum (cb). GlyT1 is in addition expressed in eye (ey) olfactory bulb (ob) forebrain regions (fb), liver (li), pancreas (pa) and in the gastrointestinal tract (git). (Adapted from Gomeza et al., 2006)

1.3 TREATMENT REGIME WITH VALPROATE, SODIUM BENZOATE AND DEXTROMETHORPHAN

The effect of NKH therapy with sodium benzoate and dextromethorphan on glycine levels due to GlyT1 has not been evaluated to date, therefore, this is the first study to study to evaluate effect of NKH treatment in expression of GlyT1. Taking in consideration the debate on the effectiveness of the NKH treatment, factors such as metabolism needs to be considered for each compound. Metabolism modifies compounds in ways possible to sustain cell vitality. These compounds are bio transformed via a two phase reaction (phase I and phase II) (Jancova et al., 2010). During phase I, drug metabolizing enzymes expose the polarity of the compound and in phase II, the enzymes conjugate the compounds. Consequently, polarization of these compounds will result in effortless excretion (Jancova et al., 2010).

In relation to NKH development, certain therapeutic drugs have been reported to either induce hyperglycinemia (valproate) (Viljoen et al., 2012), or lower the concentration of glycine in blood, urine and CSF (sodium benzoate) (Neuberger et al., 2000, Van Hove et al., 2005), or antagonise the function of NMDA (dextromethorphan) (Hamosh et al., 1990, Randak et al., 2000, Canning, 2009). It will be evident that the inadequate metabolism of these compounds might result in ineffective treatment.

1.3.1 Valproate metabolism

Valproate (sodium valproate) is a simple branched-chain carboxyclic acid that differs markedly in structure from other antiepileptic drugs (Löscher, 1999, Stapleton et al., 2008). It has unclear mechanism of action (Johannessen, 2000) and is widely used as an anticonvulsant drug to treat epilepsy, bipolar diseases, migraine and neuropathic pain (Cipriani et al., 2013, Gill et al., 2011). It is known to elevate glycine levels thus resulting in secondary NKH. Evidence shows that valproate has a participating role in increasing the levels of gamma-aminobutyric acid (GABA) in the brain (Hung et al., 2011, Gill et al., 2011). It also affects several enzymes connected to the synthesis and degradation of GABA and metabolites.

Valproate is almost entirely metabolised by the liver (bioavailability >80%). The half-life differs between species and is reported to be 2-5 hours in rats, 0.8 hours in mice, 0.6 hours in the rhesus monkey and 9-18 hours in the human (Lagace et al., 2004). Valproate is metabolised by the liver and approximately 30-70% of the administered dose is excreted in the urine as glucuronide conjugate (Hung et al., 2011, Guo et al., 2012). The biotransformation of valproate is known to occur in three major pathways namely 1) the uridine 5’ diphosphoglucuronosyl-transferase (UGT) pathway, 2) beta oxidation and 3) the cytochrome P450 (CYP450) pathways. However, glucuronidation (50%) and beta oxidation (40%) are considered the major

routes of valproate metabolism (Ghodke-Puranik et al., 2013). Glucuronidation is a phase II biotransformation reaction that results in an upsurge in polarity to create water soluble substances (Guillemette, 2003). Changes in valproate glucuronidation reportedly result in blood level variations causing insufficient treatment (Chatzistefanidis et al., 2012).

1.3.2 Sodium benzoate metabolism

In contrast to valproate, sodium benzoate is commonly used as preservatives in pharmaceuticals, cosmetic, preserved food and drinks (Monanu et al., 2005, Afshar et al., 2013). It is a preferred preservative that is soluble in water compared to ethanol (Wibbertmann et al., 2000, Shahmihammadi et al., 2016). Sodium benzoate converts to benzoic acid under acidic conditions such as in the stomach (Wibbertmann et al., 2000). After oral administration, sodium benzoate is absorbed from the alimentary canal, metabolized by the liver and excreted as hippurate in urine (Van Hove et al., 2005). Treatment with sodium benzoate is directed towards reducing glycine levels and antagonizing the action of glycine at neurotransmitter receptors such as NMDA (Van Hove et al., 2005, Misel et al., 2013). Doses of sodium benzoate exceeding 750 mg/kg/day are associated with toxicity (Bergeron et al., 1998) and results in adverse gastrointestinal effects such as nausea, vomiting, and burning sensation upon swallowing.

The mechanism of action for sodium benzoate is guided by glycine N-acyltransferase (GLYAT), an enzyme responsible for glycine conjugation with sodium benzoate (Badenhorst et al., 2013). The glycine conjugation process involves two stages ensuing hippurate formation (Figure 1.7). During the first stage, sodium benzoate is activated to benzyl-CoA by co-ligase in the presence of ATP through GLYAT in the mitochondria of the liver and kidney (Gregus et al., 1998). Thereafter, hippurate is formed after elimination of CoA and excreted in urine by

glomerular filtration and tubular secretion (Kubota and Ishizaki, 1991, Beyoglu and Idle, 2012, Lennerz et al., 2015).

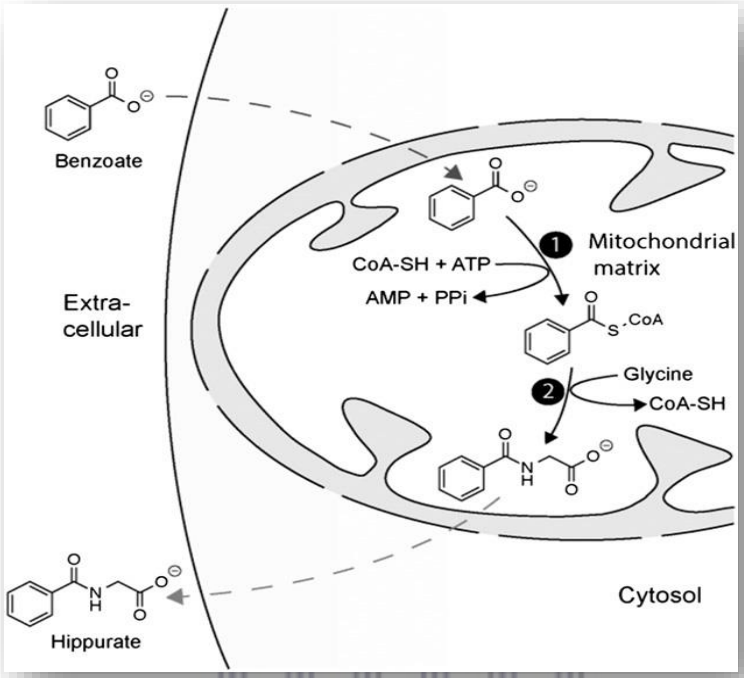


Figure 1.7: Metabolism of benzoate in the mitochondria (Lennerz et al., 2015).

1.3.3 Dextromethorphan metabolism

Dextromethorphan is a antitussive drug used to temporarily relieve cough by affecting the signals in the medulla oblongata that trigger cough reflex (Siu and Drachtman, 2007, Bem and Peck, 1992). On account of its pharmacological benefits, dextromethorphan is broadly used as an anticonvulsant, a neuroprotectant, an anti-ischaemic and an analgesic drug. (Ilkjaer et al., 1997). The properties of dextromethorphan are attributed to its ability to antagonize NMDA receptors (Franklin and Murray, 1992, Boyer, 2004, Burns and Boyer, 2013)

Dextromethorphan is absorbed from the gastro-intestinal tract and has a plasma half-life of 2-4 hours (Pender and Parks, 1991, Boyer, 2004) with a duration action of 3-6 hours after

ingestion (Levine, 2007). After absorption, dextromethorphan is metabolized to its O-demethylated metabolite (dextrorphan) for elimination through cytochrome P450 (CYP2D6) (Schadel et al., 1995, Rodrigues et al., 2008, Burns and Boyer, 2013) unchanged or as demethylated product (Siu and Drachtman, 2007). Dextrorphan is the major active form of dextromethorphan (Franklin and Murray, 1992, Boyer, 2004) that produces neurobehavioral effects (Burns and Boyer, 2013). It has been reported that both dextromethorphan and dextrorphan are responsible for psychoactive effects (Reissig et al., 2012). The mechanism of action and the effects of the combined use of dextromethorphan and sodium benzoate is unknown in non-human primates (NHP).

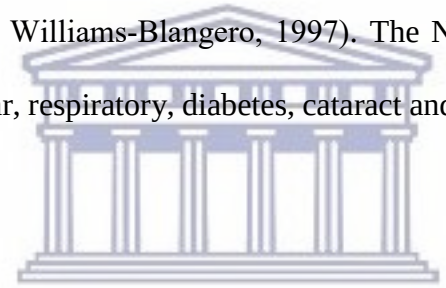
Therefore, valproate, sodium benzoate and dextromethorphan are associated with hyperglycinemia by either inducing or normalising glycine levels in the NHP model. The data accumulated in this study will contribute to the field of primatology thus making the NHP an alternative model to study NKH.

1.4 THE CAPTIVE-BRED VERVET MONKEY AS AN ANIMAL MODEL FOR HYPERGLYCINEMIA

Among the laboratory animals, NHPs are excellent models to study human biology and related diseases. They are commonly used because of their close phylogenetic relation with humans that is derived from their common ancestry (Capitanio and Emborg, 2008). Humans and NHPs share many characteristics such as evolutionary history, brain development, anatomy, physiology, metabolism, and genomics (King et al., 1988, VandeBerg and Williams-Blangero, 1997, Jasinska et al., 2013, Harding, 2013, Capitanio and Emborg, 2008). Moreover, NHPs are suitable animal models for genetic studies as they share specific genetic mechanism, have highly conserved gene maps, high degree of genetic similarities and have susceptibility to

pathogens affecting humans (VandeBerg and Williams-Blangero, 1997). In addition to genetic characters, NHPs have additional features similar to humans such as complex brain structures, complex cognitive capabilities, diet, social structure, and have susceptibility to major pathogens (Bauman et al., 2013, Jasinska et al., 2013, Capitanio and Emborg, 2008).

Conversely, it is advantageous to use NHPs rather than humans because they can be controlled experimentally, environmentally, genetically and are easily accessible for sampling (VandeBerg and Williams-Blangero, 1997). In many basic and applied studies NHPs are useful when studying diseases or disorders that affect humans (Glogowski et al., 2012, Rogers et al., 2006), or when they possess behavioural characteristics which are similar to the disease being investigated (VandeBerg and Williams-Blangero, 1997). The NHP model has been used to study HIV/SIV, cardiovascular, respiratory, diabetes, cataract and nonketotic hyperglycinemia etc.



Among the African primates, rhesus and vervet are the old world NHPs commonly used for biomedical studies. Vervet monkey has been recognised as the best alternative for rhesus as a result of limited availability of the latter species (Jasinska et al., 2013, Freimer et al., 2011). Vervet monkeys offer a valuable experimental model for genetic and genomic investigations in relation to human diseases, thus making the vervet monkey an important resource for biological studies (Jasinska et al., 2013). However, the availability and cost to maintain NHPs prevent most researchers from using them as animal model especially in captivity where their wellbeing, environment and care is important (Liddie et al., 2010).

At PUDAC, vervet and rhesus monkeys have been extensively used for biomedical studies such as cardiovascular disease (cholesterol transport, atherosclerosis), reproduction (male and

female contraception), vaccine development, drug development (pharmacokinetics, pharmacodynamics), metabolic diseases (diabetes and obesity) etc. As previously mentioned, spontaneous congenital cataract in this specific colony of captive bred vervet monkeys, have previously been described (de Villiers et al., 2001). Consequently, cataract formation and the possible connection to hyperglycinemia in affected individuals, provided an opportunity to investigate the molecular basis of NKH in the vervet model by understanding the dynamics of hyperglycinemia. This was achieved by using intervention approach (Pharmacodynamics and clinical biochemistry) and molecular genetic approach (bioinformatics, genotyping and gene expression).

This is the first study to report on the primary genetic dynamics of NKH in the NHP model. The occurrence of this disorder in this specific colony of captive-bred vervet monkeys, presented an opportunity to investigate to further expand our knowledge on the validation of the vervet monkey as biomedical research model. The findings of this study will contribute to the understanding of the genetic mechanism of NKH.

1.5 RESEARCH AIMS AND OBJECTIVES

The main focus of the study was to determine the underlying cause of hyperglycinemia in vervet monkeys and to normalise glycine levels by administering sodium benzoate and dextromethorphan. Thereby making an original contribution to primatology by defining the molecular genetics of captive-bred vervet model in relation to NKH.

Primary objectives

- The aim of this study was to investigate NKH by studying the condition in a spontaneous hyperglycinemic and an induced hyperglycinemic vervet model.

- The second aim was to determine the pharmacodynamics of compounds such as valproate, sodium benzoate and dextromethorphan in relation to gene expression.
- Finally, to understand further the inter-individual variation in single gene interactions.

Secondary Objectives

- To determine the level of glycine in urine, plasma and cerebrospinal fluid in monkeys with spontaneous hyperglycinemia, as well as in monkeys with valproate induced hyperglycinemia.
- To determine if valproate-induced hyperglycinemia can be reversed by sodium benzoate and dextromethorphan treatment.
- To screen for polymorphism/sequence variants that are associated with valproate metabolism and glycine transportation.
- To determine the effect of valproate, sodium benzoate and dextromethorphan at genetic level by the expression profile of the selected cataract and NKH genes.

1.6 RESEARCH HYPOTHESIS

The available NKH treatment is effective in reducing glycine in NHP as it is in human patients. The underlying genetic factors associated with NKH and cataract in vervet monkeys are similar to those found in humans, therefore, genes that are implicated in cataract and NKH development in humans are considered candidate genes in the vervet monkey.

1.7 RESEARCH OUTPUTS

Findings reported here were presented at various scientific conferences in 2014-2015

Conference presentations:

1. “Hyperglycinemia in cataract captive bred vervet monkeys (*Chlorocebus aethiops*), Novartis (June 2014, Basel, Switzerland). Oral presentation.
2. “Effect of valproate, sodium benzoate and dextromethorphan in hyperglycinemic captive bred Vervet monkeys (*Chlorocebus aethiops*)”. Presented in the following local and international conferences:
    - 13th International Congress of the European Association for Veterinary Pharmacology and Toxicology (EAVPT) conference (July 2015, Nantes, France). Poster presentation
    - 3rd International Conference on Genomics & Pharmacogenomics (September 2015, San Antonio, USA). Poster presentation
    - 9th Annual Early Career Scientist Convention at SAMRC (October 2015, Cape Town, South Africa) Oral presentation
    - Research Open Day at UWC (October 2015, Bellville, South Africa). Poster presentation
    - The South African Annual Pharmacology Conference (SAPHARM-2017) at the University of Free State (01-04 October 2017, Bloemfontein, South Africa). Poster presentation
    - The SAALAS (South African Association for Laboratory Animal Science) International Conference (01-03 November 2017, Stellenbosch, South Africa)

CHAPTER TWO:

Materials and Methods

2.1 ETHICAL APPROVAL

The study was conducted in accordance with the Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals (A5726-01). The research protocol (Ref.08/13) was approved by the Ethics Committee for Research on Animals (ECRA) of the South African Medical Research Council (SAMRC). All procedures for sample collection and animal housing were in accordance to the ethical guidelines of South African National Standard for the Care and Use of Animals for Scientific Purposes (The SANS 10386:2008).

2.2 ANIMAL INTERVENTION AND EXPERIMENTAL DESIGN

The animal intervention focused on therapeutic administration of valproate, sodium benzoate and dextromethorphan. Pharmacodynamics of these compounds was determined using blood samples, which were later used for genotyping and gene expression discussed in chapter four and five. All selected individuals were housed indoors in single cages for the duration of the study with regular access to exercise cages. The closed indoor environment was maintained at a temperature of 24-26°C, 40-70% humidity, 15-20 air changes/hour and a photoperiod of 12 hours. All diets were prepared at PUDAC and all subjects were fed three times a day. The maintenance diet consisted of pre-cooked maize meal mixed with a vitamin and mineral concentrate, egg powder, bean flour and sunflower oil and supplemented with vitamin C and D3. An additional fruit portion was offered mid-afternoon. As enrichment, a mixture of ground and whole kernel maize was later provided to allow foraging. Water was available *ad lib* via an automatic watering device.

2.2.1 Subject selection

Vervet monkeys were monitored for cataract formation with related glycine levels exceeding the normal reference in CSF (>10 µmol/L). Thereafter, the animal intervention study was divided into two phases and twelve monkeys were assigned into three groups: control, induced and spontaneous (Table 2.1). The control and induced groups did not have cataract and glycine levels in CSF were normal, whereas the spontaneous group was presented with both cataract and hyperglycinemic. Four of the healthy monkeys were assigned to a control group and received a maintenance diet throughout the study. In phase one, four healthy monkeys were induced with valproate (50 mg/kg/day) for three weeks to increase glycine levels. In phase two, four spontaneous monkeys were treated with sodium benzoate (250 mg/kg/day) and dextromethorphan (5 mg/kg/day) for four weeks collectively with the induced group that was used in phase one. The effective dosages were chosen for each drug based on previous human studies (Arnold et al., 1997, Van Hove et al., 2005, Viljoen et al., 2012). The reported effective dose for dextromethorphan is 5-35 mg/kg/day and sodium benzoate 250-750 mg/kg/day (Hamosh et al., 1992, Schmitt et al., 1993). The washout period served as a withdrawal period for drug intervention.

Table 2.1: Group selection and treatment allocation

GROUP	TREATMENT	DOSES mg/kg	No. of monkeys	Treatment time	Duration
1:Control	Maintenance diet	n/a	4	7:30am	Throughout
2:Induced	Valproate	50 mg/kg/day		7:30am	3 weeks
	Sodium benzoate and	250 mg/kg/day	4	7:30am	4 weeks
	Dextromethorphan	5 mg/kg/day			
3:Spontaneous	Sodium benzoate and	250 mg/kg/day	4	7:30am	4 weeks
	Dextromethorphan	5 mg/kg/day			

2.2.2 Compound formulations and administration

Valproate and sodium benzoate (Sigma Aldrich, South Africa) were administered in a powder form and dextromethorphan (Johnson & Johnson, South Africa) was provided as syrup. The dosages for valproate (50 mg/kg), sodium benzoate (250 mg/kg) were weighed and dextromethorphan was measured (5 mg/kg) accordingly (Table 2.2) and mixed to a 30 g portion of food (pre-cooked maize meal). The treated food was administered once daily in the mornings and was consumed voluntarily by animals. The rest of the food (70 g) was offered once the treated food was consumed and food intake was recorded daily. This is the standard method which has been well documented in PUDAC's standard operating procedures (Chauke et al., 2015).

Table 2.2: Baseline drug intervention to determine the efficacy of valproate, sodium benzoate and dextromethorphan in vervet monkeys

Groups	Animal ID	Weight baseline	Baseline CSF	Baseline plasma	VPA(Kg)	SD (Kg)	DX (ml)
Control	399	3.6	7.8	455	0	0.00	0.00
	145	3.69	9.1	460	0	0.00	0.00
	127	5.57	8.9	470	0	0.00	0.00
	178	3.85	8.4	528	0	0.00	0.00
Induced	338	3.26	7.5	589	0.16	0.82	5.43
	104	3.82	7.1	785	0.19	0.96	6.37
	113	3.29	7.2	246	0.16	0.82	5.48
	438	2.18	8.5	486	0.11	0.55	3.63
Spontaneous	409	3.04	12.7*	532	0	0.76	5.07
	416	3.05	10.6*	498	0	0.76	5.08
	412	3.39	11.02*	605	0	0.85	5.65
	398	5.2	11.5*	579	0	1.30	8.67

VPA=valproate, SD= sodium benzoate, DX=dextromethorphan and *= above normal levels (>10)

2.2.3 Duration of animal intervention

The duration of the study was 13 weeks which included four time points; baseline (two weeks), phase one (valproate), phase two (sodium benzoate and dextromethorphan) and washout period (four weeks) (Table 2.3).

Table 2.3: Experimental layout

	#Animals	Baseline	Phase 1	Phase 2	Washout
Groups	**Period**	2 weeks	3 weeks	4 weeks	4 weeks
Control	4	Blood, urine & CSF Baseline T0	Blood, urine & CSF T1	Blood, urine & CSF T2	Blood, urine & CSF washout T3
Induced	4				
Spontaneous	4				

2.3 SAMPLE COLLECTIONS

2.3.1 Blood collection

The animals were sedated with Ketamine hydrochloride (10mg/kg) (Kyron laboratories, South Africa) anaesthesia two hours after compound administration and blood samples were obtained via femoral venepuncture. On each day of sampling, monkeys received the total dose of the compound (Table 2.1) in small food balls and blood was taken two hours after compound administration. Blood (2-4ml) for biochemistry analysis and plasma isolation was collected in ethylenediaminetetraacetic acid (EDTA)-containing tubes. Plasma was isolated by centrifugation at 4°C (1400 x g, 10 min) and thereafter maintained at -80°C for glycine analysis.

Blood (2.2ml) for gene expression was collected in PAXgene Blood RNA Tubes (BRT) (PreAnalytiX, Qiagen) and stored at room temperature for two hours before being stored at -80°C. For clinical biochemistry and haematology, 2 ml of blood was collected into SST,

Sodium Fluoride/Potassium Oxalate and EDTA tubes to perform clinical biochemistry and haematology tests. The clinical biochemistry included test for alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatise (ALP), gamma-glutamyl transferase (GGT), total protein, bilirubin (total and direct), albumin, globulin, cholesterol (total, LDL-C, HDL-C), urea, creatinine, triglyceride, calcium (Ca), sodium (Na), magnesium (Mg), chloride (Chl), anion gap, phosphate (P), potassium (K), total bicarbonate (CO2) and creatine kinase (CK). Complete blood count examination included red blood cells (RBC), total white blood cells (WBC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH); mean corpuscular haemoglobin concentration (MCHC), haematocrit (HCT), red blood cell distribution (RDW), haemoglobin (Hb), neutrophils, basophils, lymphocytes, monocytes and platelets count.

2.3.2 Cerebrospinal fluid (CSF) collection

Cerebrospinal fluid (CSF) was collected while animals were still sedated with ketamine hydrochloride (10mg/kg) at baseline, three weeks after phase one, at the end of phase two and after washout period. Firstly, the back of the animal's head was shaved and disinfected. The monkey was held by the operator to minimise movements with the head flexed ventrally as far as possible. A gauge was inserted in the cisterna magna to aspirate the sample and thereafter collected into a sterile 1.5ml eppendorf tube. The syringe was removed and pressure was applied to the area of puncture. The sample was placed immediately on ice and stored at -80°C for glycine analysis.

2.3.3 Urine collection

Urine (24 hour) samples were collected after the morning feeding. A stainless-steel collection pan was placed underneath each cage. Each collection pan was fitted with a gritted funnel to

prevent food and faeces contamination. Urine was then directly collected via the funnel into a sterile bottle.

2.4 CLINICAL OBSERVATIONS

During sampling days, special observations were followed and animals were monitored after CSF collection. The weight of each animal, body temperature, heart and respiration rate and blood pressure were recorded. Food intake was observed daily and recorded, while general well-being was observed daily and recorded weekly. These included posture, coordination, locomotion, activity, behavioural changes (such as: alertness, fearfulness, aggressiveness, confusion, depression, vocalisation), discharge from orifices, appetite, condition of faeces and urine. Observations were recorded on log sheets specifically designed for this purpose (Appendix A).

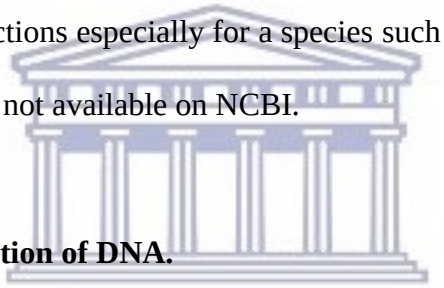
2.5 GENOTYPING FOR SELECTED GENES

Five genes were retrieved, screened, and genotyped, sequenced and expressed using bioinformatics tools which will be discussed in detail in chapter four and five. Among these genes, *GLDC* and *AMT* were adopted from a previous in-house project which focused on the identification of sequence variants causing hyperglycinemia (Chauke et al., 2016), therefore, only gene expression was investigated in this study. The remaining genes consist of *UGT1A6*, *UGT1A9* and *SLC6A9*.

2.5.1 Identification of candidate genes and genotyping

The human genes that are known to affect valproate glucuronidation (*UGT1A6* and *UGT1A9*) and the GlyT1 (*SLC6A9*) were prioritized for genotyping in vervet monkeys. These genes were located within the previously published public domain databases, containing annotated genes

(NCBI GENBANK: http://www.ncbi.nlm.nih.gov/), as well as by applying gene prediction programs (ENSEMBL). Sequences for *UGT1A6* (accession no: NM_001072.3), *UGT1A9* (accession no: NM_021027.2) and *SLCA9* (accession no: XM_002801515.1) were retrieved from NCBI database. Due to unavailable vervet genome sequence, the rhesus macaque was used as an alternative model to design primers (Appendix B, Table B1), since both species belong to the same superfamily (Old World monkeys). The UCSC genome browser (http://genome.ucsc.edu) was utilized to blast human sequence against the rhesus macaque. Tools such as ClustalW (http://www.genome.jp/tools/clustalw/) and NCBI were used for cross-species sequence comparisons. Comparing the genomic sequences of species at different evolutionary distances assist in identifying coding sequences and conserved noncoding sequences with regulatory functions especially for a species such as the vervet monkey where the whole genome sequence is not available on NCBI.

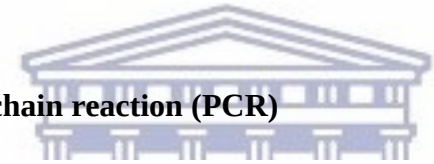


2.5.2 Extraction and purification of DNA.

AxyPrep Blood Genomic Deoxyribonucleic acid (DNA) Miniprep kit (Axygen Biosciences, USA) was used to extract DNA from whole blood of selected vervet monkeys (Appendix B3). Blood genomic DNA is directly isolated from the white blood cell (WBC) component of whole blood, without the need to remove the red blood cells (RBCs) in advance. The procedure of extracting DNA was performed according to the manufacturer's instructions. Maintaining the integrity and reactivity of the genomic DNA, particularly in polymerase chain reaction (PCR), the purified genomic deoxyribonucleic acid (DNA) was eluted and stored in low-salt Tris buffer containing 0.5-1 mM EDTA.

Briefly, anti-coagulated blood was mixed with buffer AP1 (cell lysis buffer) by vortexing at top speed for 10 seconds. Subsequently, buffer AP2 was added, mixed for 10 seconds and the

mixture was centrifuged at 12,000 x g for 10 minutes at ambient temperature to pellet cellular debris. Binding was performed by applying the clarified supernatant to the Miniprep column and centrifuged at 6, 000 x g for one minute. The bound DNA was washed with buffer W1A (wash buffer). The Miniprep column was allowed to stand at room temperature for two minutes and centrifuged at 6,000 x g for one minute. Desalting was performed twice using buffer W2 (desalting buffer) followed by centrifugation at 12,000 x g for one minute. Thereafter, the Miniprep column was centrifuged at 12,000 x g for one minute to get rid of residual solutions. Finally, DNA was eluted by adding the pre-warmed (at 65°C) TE buffer and the mixture was allowed to stand at room temperature for one minute. Thereafter, centrifuged at 12, 000 x g for one minute and the eluted DNA was stored at -20°C.



2.5.3 Standard polymerase chain reaction (PCR)

Selected genes were amplified by polymerase chain reaction (PCR) which is an *in vitro* enzymatic amplification of defined DNA sequences to produce a high yield of amplified target DNA. This occurs under the influence of specifically designed primers and a thermostable DNA polymerase of *Thermus aquaticus* (Taq). The process consists of three distinct steps. The first is denaturation of double stranded DNA followed by annealing of the primers to their complementary sequences on the template. The final step is extension by incorporation of nucleotides under the influence of Taq polymerase. With numerous repetitions of this set of steps, the number of copies of the target sequence rises exponentially.

In order to amplify target DNA, 0.2 ml thin walled tubes were used in a G-storm gradient thermocycler S/N 20024 (Vacutec, SA) equipped with a heated lid. Unless otherwise stated, the standard 25 µl-PCR reactions (Table 2.4) contained the following reagents: 2x PCR Master

Mix (Promega, USA), DNA template (50 ng) and 0.5 µM of the upstream and downstream primers were made up to 25 µl with Nuclease-Free water.

Table 2.4: PCR sample preparation

Components (Promega)	Experiment (µl)	Control (µl)	Final concentration
Master mix 2X	12.5µl	12.5µl	1X
Primers (R&F) 10µM	1.25µl x2	1.25µl x2	0.5µM
DNA	1µl	0µl	50ng
Nuclease free water to	25µl	25µl	N/A

The PCR conditions were performed at: 95ºC for five minutes followed by 30 cycles of 95ºC for 30 seconds, XºC for 30 seconds and 72ºC for one minute; an extension period of five minutes at 72ºC completing the procedure. X denotes the relevant annealing temperature which was chosen 5ºC below the assumed primer melting temperatures calculated using the following formula (Tm= [no. of GC] x 4 + [no. of AT] x 2ºC (Table 2.5)

Table 2.5: PCR conditions

Step	Temperature (°c)	Time	Cycle
Initial denaturation	95	5min	1
Final denaturation	95	30sec	
Annealing	X (primer dependant)	30sec	30
Elongation	72	1min	
Final elongation	72	5min	1

2.5.3.1 Electrophoresis

Nucleic acids were separated by agarose gel electrophoresis technique based on charge, size and conformation. DNA is negatively charged and migrate away from the negative pole through the agarose gel to the positive pole when the gel is placed in buffer in an electrical

field. The molecules separate out with the larger and more folded molecules moving slower through the gel and settling closer to the origin than the smaller molecules that are able to move faster through the gel. Lastly, DNA ladder (GeneDireX, USA) with known band sizes is used on all gels as reference to determine the unknown target DNA.

2.5.3.2 Agarose gel

Agarose gels were made by dissolving 0.5-1g of agarose in 1X tris borate EDTA (TBE) buffer (Appendix B4-8) which was made from 5X TBE buffer for 0.8 - 2% gels, depending on the fragment size loaded onto the gel. Genomic DNA was run on 1% gels, whereas 1.5-2% gels were used for fragment sizes of 50 bp and 2000 bp. The agarose gels were running in TBE buffer at a voltage range between 100 - 120 V for approximately 30 minutes. Samples were loaded into the wells with 10% tracking dye (Appendix A8). 0.5 mg/µl ethidium bromide (EtBr) was added to the gels to allow visualisation of DNA when it was placed on an ultra violet transilluminator, which caused any DNA bound to ethidium bromide to fluoresce. A 100bp DNA ladder was used as a marker to identify the molecular weight of amplified exons.

2.5.3.3 Purification of PCR products.

The PCR products were purified in order for the samples to be suitable for sequencing reactions. This was achieved using Wizard® SV Gel and PCR clean-up system (Promega, USA) according to the manufacturer's instructions (Appendix B9). This method is based on the ability of DNA to bind to silica membranes in the presence of chaotropic salts. After electrophoresis, an aliquot of the PCR is added to the Membrane Binding Solution and directly purified. The system allows a choice of methods for isolation of DNA from the dissolved agarose gel slice or PCR amplification. DNA can be isolated using microcentrifugation (Eppendorf centrifuge 5415 R) to force the dissolved gel slice or PCR product through the

membrane while simultaneously binding the DNA on the surface of the silica. After washing the isolated PCR product, the DNA is eluted in water.

2.5.4 Sequencing reactions

The purified PCR samples were sequenced for mutation analysis. Sequencing was performed using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer) (Appendix B, Table B2). The reagents were vortexed then spun briefly before the PCR sequencing reaction (Appendix B, Table B3) was started.

2.5.5 DNA sequence analysis

Sequencing data was analysed using the CLC Main workbench 6.1.1 (CLC Bio, Denmark) to identify the sequence variants within the selected colony. Translation of DNA to protein sequence was achieved using both CLC DNA workbench and ExPASy translate tool (http://web.expasy.org/translate/). ClustaW was used for aligning DNA and protein sequences to elucidate their relatedness and by checking for conserved regions.

2.6 GENE EXPRESSION

Quantitative real-time PCR (qRT-PCR) is currently the most precise method to measure and quantify messenger RNA (mRNA) in biological samples. An amplification product can be detected and measure simultaneously during each PCR cycle. In this study, RT^2 SYBR® Green qPCR gene expression assays (Qiagen, Germany) were used to quantify selected genes. SYBR Green is a fluorescent dye which emits a fluorescent signal upon binding to any double-stranded DNA. It is the most economical qRT-PCR chemistry and easy to design. However, SYBR green will bind to any double-stranded DNA including primer dimers or contaminated DNA, therefore, a melting curve is necessary to ensure specificity

2.6.1 RNA extraction from whole blood

The PAXgene tubes have been shown to reduce RNA degradation in whole blood samples. The tubes contain a reagent that immediately stabilizes RNA by protecting its degradation by RNases and minimized ex vivo changes in gene expression. The PAXgene Blood RNA Kit (Qiagen, Switzerland) was used to isolate and purify intracellular RNA from whole blood collected in the PAXgene Blood Tube (BRT) during animal intervention.

The PAXgene tubes were centrifuged to pellet the nucleic acids and thereafter the pellet was re-suspended with RNase free water. The re-suspended pellet was incubated in optimised buffer with proteinase K collectively to initiate protein digestion. An additional centrifugation through the PAXgene shreder spin column was carried out to homogenise the cell lysate and remove residual cell debris. The supernatant of the flow-through fraction was transferred to a fresh micro-centrifuge tube. Ethanol was added to adjust binding conditions, and the lysate was applied to a PAXgene RNA spin column. During a brief centrifugation, RNA was selectively bonded to the PAXgene silica membrane as contaminants pass through. Remaining contaminants were removed in several efficient wash steps using PAXgene Blood RNA Kit (Qiagen, Germany). The RNA was eluted in elution buffer, heat-denatured and immediately placed on ice.

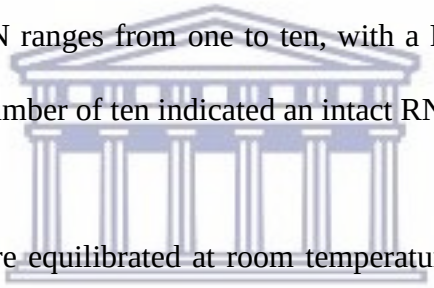
2.6.1.1 Measuring RNA concentration

The concentration of extracted RNA was measured using a NanoDrop 2000 UV-visible spectrophotometer (Vacutec, SA). The NanoDrop software displays the concentration in ng/µl and an estimation of the purity of RNA is assessed by 260/280 ratio. The ratio of 260/230 is used as a secondary measure of purity, and indicates co-purified contaminants. A ratio of 1.8 - 2.0 is accepted as pure for RNA. The spectrophotometer was initialised by pipetting 2 µl of

RNase-free water onto the pedestal of the spectrophotometer and the blank option was selected. Thereafter, 2 µl sample was pipetted onto the pedestal and the absorbance determined. Each sample was recorded in triplicates.

2.6.1.2 Measuring RNA integrity (RIN)

RNA integrity number (RIN) was determined with the Agilent 2100 bioanalyzer (Agilent technologies, Germany) in conjunction with the RNA 6000 Nano-kit according to the manufacturer's instructions. The software assigns an RIN to the RNA sample. The RIN is a numerical assessment of the integrity of the RNA sample, taking into account the entire electrophoretic pattern of the RNA sample (28S:18S ratio) and the presence or absence of degradation products. The RIN ranges from one to ten, with a RIN of one indicating totally degraded RNA while a RIN number of ten indicated an intact RNA sample.



The reagents and samples were equilibrated at room temperature for 30 minutes before use (according to the Agilent Bioanalyzer protocol). The electrodes were decontaminated by washing with RNAse ZAP for one minutes and with RNAse free water for 10 seconds. The red Agilent Nano gel matrix (550 µl) was added to the spin filter and centrifuged for 10 minutes at 5000 rpm. Thereafter, 65µl of the filtered gel was aliquoted in micro-centrifuge tubes and stored at 4-8°C. The gel-dye mix was prepared by adding 1µl of dye to 65 µl of filtered gel, mixed by vortexing for 10 seconds and then centrifuged at 12 000 x g for 10 minutes. The RNA 600 Nano chip was placed in the priming station and the well that is marked with dark G was filled with 9 µl of gel-dye mix and primed by pressurising the syringe in the chip priming station for 30 seconds. Thereafter, the two wells marked light G were filled with 9 µl of gel-dye mix. The RNA 6000 Nano marker (5 µl) was added to the 12 sample and one ladder well. The RNA ladder and samples (50-500 ng/µl) were denatured by placing them on the incubator

at 70°C for two minutes. Then, 1 µl of RNA ladder or RNA sample was pipetted into their respective wells. Then the chip was vortexed at 2400 g for one minutes and then placed in the chamber of the Agilent Bioanalyser which had been decontaminated with RNaseZap and RNase-free water. The assay was initiated after selecting the (RNA Eukaryote total RNA Nano series II) programme on the 2100 Agilent Expert software.

2.6.1.3 DNase treatment of extracted RNA

The extracted RNA samples were DNase treated to eliminate genomic DNA from RNA preparations. The TURBO DNase kit (Ambion, USA) was used according to the manufacturer's instructions. Briefly, 5 µl of 10x DNase buffer and 1.5 µl of DNase was added to 10 µg of RNA and RNase-free water in a total reaction volume of 50 µl. Samples were mixed by inverting the tube and placing it on the incubator for 45 minutes at 37°C after which another 1.5 µl of DNase was included and incubated at 37°C for a further 45 minutes. The reaction was stopped by adding 10µl DNase inactivation reagent and mixed by shaking by hand for two minutes. Thereafter, the tubes were centrifuged at 10 000 x g for 1.5 minutes and the supernatant transferred to a new tube. The RNA concentrations were determined using a Nanodrop 1000 spectrophotometer.

2.6.1.4 Reverse transcription (RT) of RNA to cDNA

Complementary DNA (cDNA) was synthesised from total RNA using High-Capacity cDNA kit according to the manufacturer's instructions (Applied Biosystems, USA). Reverse transcriptase is an enzyme that translates RNA to DNA. This creates one DNA (cDNA) strand complementary to the RNA strand in a DNA-RNA hybrid. One microgram DNase treated RNA sample was added to a final volume of 10 µl RNase-free water and placed on ice. A reaction mix consisting of reaction buffer, dNTPs, random primers, RNase-inhibitor (5000 units/ml),

reverse transcriptase and nuclease-free water was prepared into two separate tubes labelled RT plus and RT minus. The RT minus reaction mix tube (negative control) contained the same reaction mix as the RT plus tube, but with the reverse transcription enzyme replaced by water (Table 2.6). The RT minus tube was included to determine the genomic DNA contamination. After adding the RT plus and RT minus mix components, the prepared reaction were mixed by repeated pipetting and the tubes were centrifuged briefly. Ten microliters of plus or minus RT reaction mixes were added to 0.2 ml tubes containing RNA samples to a final volume of 20 µl. The tube contents were mixed, briefly centrifuged and placed in a G-storm thermal cycler. The reactions were incubated for three cycles: 1) at 25°C for 10 minutes, 2) 37°C for 120 minutes and 3) 85°C for five minutes to inactivate the reverse transcriptase enzyme at 25°C for 10 minutes, 37°C for 120 minutes, and 85°C for 5 minutes to inactivate the reverse transcriptase enzyme. Samples were stored at -20°C for later gene expression analysis.

Table 2.6: Reaction components for the reverse transcription reaction

Component	Volume (µl)	
	Plus RT	Minus RT
1 µg DNase-treated RNA in RNase-free	10	10
10 x RT buffer	2	2
25 x dNTP mix	0.8	0.8
10 x random primers	2	2
RNase inhibitor	1	1
Nuclease-free water	3.2	4.2
Reverse Transcriptase	1	_
Total volume	**20**	**20**

2.6.1.5 Standard PCR to confirm cDNA conversion

To assess the extent of genomic DNA contamination in RNA samples, cDNA generated from plus and minus reverse transcription reactions were amplified with exon spanning primers that would amplify both cDNA and genomic DNA. A reaction mix (Table 2.7) consisting of 12.5µl

master mix, 1µl of 10µM ActB forward Primer (400 nM), 1µl of 10µM ActB reverse Primer (400nM) and H_2O to a final volume of 24µl was prepared. The reaction mix was scaled up according to the number of test samples. Thermocycling conditions were similar to the one in Table 2.5.

Table 2.7: Reaction mix to confirm genomic DNA contamination

Component	Volume (µl)	Final Concentration
2x master mix	12.5	2X
ActB Forward primer (10 µM)	1	400 nM
ActB Reverse Primer (10 µM)	1	400 nM
Water	9.5	-
cDNA	1	50 ng
Final volume	**25**	**-**

2.6.1.6 Selection of primer assays for quantitative Real-Time (qRT-PCR)

The valproate glucuronidation (*UGT1A6* and *UGT1A9*) (Table 2.8), congenital cataract (*GCNT2*), GlyT1 (*SLC6A9*) and NKH (*GLDC* and *AMT*) genes were prioritized for gene expression analysis. The aforementioned genes were selected based on their functions such as their involvement in valproate therapy, cataract development and hyperglycinemia, respectively. Phosphoglycerate kinase 1 (*PGK1*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were included as housekeeping genes to normalize mRNA levels in test samples. Therefore, the nine selected RT^2 qPCR primer assays (Qiagen, Germany) were already prepared in order to eliminate tedious primer design and assay optimization steps. These assays are bioinformatically validated, and designed for SYBR® Green based real time PCR detection. Each 10 µM RT^2 qPCR Primer Assay contains a mix of forward and reverse primers for a specific target.

Table 2.8: Selected candidate genes for mRNA expression assays

Gene	Symbol	Map position	Assay ID
1. Solute carrier family 6, member 9	*SLC6A9*	1p33	QT00068460*
2. Glucose N-acetly transferase 2	*GCNT2*	6p24	PPQ03425A**
3. Glycine decarboxylase	*GLDC*	9p24.1	PPQ15325A**
4. Aminotransferases	*AMT*	3p21.31	PPQ09444A**
5. Phosphoglycerate kinase 1	*PGK1*	Xq13.3	PPQ09326C**
6. Glyceraldehyde-3phosphate dehydrogenase	*GAPDH*	12p13	PPQ08645A**
7. UDP glucuronosyltransferase 1- polypeptide A6	*UGT1A6*	2q37	Hs_UGT1A6_1_SG*
8. UDP glucuronosyltransferase 1- polypeptide A9	*UGT1A9*	2q37	PPH01705A*

*Human primer assay and **Rhesus primer assay

2.6.1.7 Quantitative RT-PCR (qRT-PCR) amplification

Quantitative PCR was used to test various input amounts of RNA for the cDNA yield of different gene targets. The PCR was performed using RT^2 SYBR Green Rox PCR Master Mix kit (Qiagen, Germany), 7500 Real-Time PCR System SDS software (Applied Biosystems, USA), optical 96-well plates and optical adhesive covers (Applied Biosystems, USA). Universal cycling conditions; 50ºC for two minutes and 95ºC for 10 minutes, followed by 40 cycles of 95ºC for 15 seconds and 60ºC for 1 minute were used. A post-PCR amplification protocol was pre-set before the run to obtain melt curve for secondary product detection.

A 25 µl reaction mixture was prepared in 96-well reaction plates (Table 2.9). The cDNA template (≤5 ng/reaction) was added to the wells containing the reaction mixture. PCR plates were covered with adhesive film and briefly centrifuged. Thereafter, plates were briefly centrifuged at 3 000 g. The real-time cycler was then programmed and initiated as described above. After the run, default settings for the threshold cycle (Ct) and baseline were used and Ct values were exported to Microsoft Excel for analysis. Data was normalised to the average of *PGK1* and *GAPDH*.

Table 2.9: Reaction mix for qRT-PCR

PCR reaction	1X (µL)
2X RT^2 SYBR Green Rox Mix	12.5
10X Primer assay	1
cDNA	2
Water	9.5
Total volume	**25**

2.6.1.8 Generating a standard curve

A cDNA from human brain total RNA (Ambion, USA) was included in all the RT-PCR runs. The cDNA aliquots were pooled together and used to generate a standard curve, to test the monkey or human gene expression assays. A serial dilution (1:5) was prepared ranging from a stock of 50 (undiluted) to 0.08 (diluted). The reaction mix was scaled up according to the number of samples to be analysed. The reaction mix similar to Table 2.9 was aliquoted into a well of the PCR plate, followed by 2 µl of a 10-fold dilution series of the control cDNA. No-template control (NTC) using water instead of cDNA was used as a negative control in all PCR reactions, and each sample was prepared in duplicate.

To generate the standard (or calibration) curve, the threshold cycle for the standard curve reactions was plotted against the fold dilution of the template cDNA on a semi-logarithmic (base 10) plot. The fit to a straight line including the slope and the correlation factor (R^2) was determined.

2.7 STATISTICAL ANALYSIS

Data was presented as means ± SD or as means ± SEM. The MedCalc® programme version 10.4.0.0 (Mariakerke, Belgium) was used for basic statistical analyses. Tests were performed for normality of distribution. Levene's test for equality of variances was applied and when P >

0.05, one-way analysis of variance (ANOVA) analysis was performed for parametric distributions. Any significant differences ($P < 0.05$) for ANOVA analysis between groups were furthermore analysed using Dunnett's multiple comparisons test which was performed using GraphPad Prism version 6.00 for Windows (La Jolla California USA).

CHAPTER THREE:

The effect of valproate, sodium benzoate and dextromethorphan in a colony of captive-bred vervet monkeys

3.1 INTRODUCTION

The vervet monkey is a preferred NHP research model due to its small size, affordability and is easy to maintain in captivity (Freimer et al., 2011). At PUDAC, cataract monkeys with hyperglycinemia served as excellent model for this study. The onset of cataract instigated an investigation which led to the discovery of hyperglycinemia in the affected monkeys. Consequently, molecular aspect of NKH using treatment intervention with sodium benzoate and dextromethorphan were adopted. Hyperglycinemia was also mechanically induced using valproate in healthy vervet monkeys to compare the effectiveness of the treatment.

In human studies, the combination of sodium benzoate and dextromethorphan is the only available treatment that reduces glycine levels in CSF and plasma. This treatment allows some children to survive the neonatal period; it reduces the incident of seizures and alleviates severe symptoms of NKH. However, the effectiveness of the treatment also depends on the severity of the disease, the response to the treatment and different doses of the compound administered. Therefore, to achieve the desired effect, different doses of sodium benzoate and dextromethorphan must be adjusted according to each NKH affected case (Van Hove et al., 2005, Lu et al., 1999, Zammarchi et al., 1994).

During drug intervention, the efficacy and mode of action for each compound must be known as various metabolic and detoxification reactions occur in the liver (Padda et al., 2011). It is therefore indispensable to continuously test for drug adverse effect using haematology and

clinical biochemistry. These parameters assist in determining meaningful predictive values, the wellbeing of each individual and can be monitored throughout the study (Kagira et al., 2007, Liddie et al., 2010). In captive-bred vervet monkeys, factors such as captivity conditions, sex, age, diet and stress are reported to have a direct impact on the haematology and biochemical parameters (Castro et al., 2015). Therefore, baseline parameters must be recorded before commencing with drug intervention (Kagira et al., 2007, Castro et al., 2015), as these can be an indication of compound efficacy.

Amongst these biochemical parameters, aminotransferases (ALT and AST) serve as general markers for liver function and are used to assess liver damage (Moss and Rosalki, 1996, Giannini et al., 2005, Wu et al., 2014). Patients with elevated aminotransferases are usually diagnosed with hepatocellular injury (Giannini et al., 2005), however, ALT elevation can also be connected to heart or skeletal muscle damage (Aziz and Zabut, 2012). Conversely, alkaline phosphatase (ALP) transports metabolites across the cell membrane of intestine, liver, bone, spleen and kidney. A high level of ALP gives an indication of liver and bone malfunctions (Sturgill and Lambert, 1997, Aziz and Zabut, 2012, Giannini et al., 2005, Wu et al., 2014). Additionally, if ALP is elevated and gamma-glutamyl transferase (GGT) is not, that points to extrahepatic origin (Ahmed and Siddiqi, 2006). Valproate and sodium benzoate have been reported to have an impact on parameters such as ALT, AST, ALP, platelet count, total protein and albumin (Ibekwe et al., 2007, Chen et al., 2012, Hauser et al., 1996, Amrani et al., 2013). Therefore, it is possible that both compounds will have a similar effect in nonhuman primates.

Therefore, the purpose of this chapter is to outline the effect of valproate, sodium benzoate and dextromethorphan on glycine levels, biochemical and haematological parameters. The animal intervention approach was a preparation for the second phase of the study that focused on

genotyping and gene expression of prioritized genes as previously mentioned in chapter two (*UGT1A6, UGT1A9, SLC6A9, GLDC* and *AMT*).

3.2 MATERIALS AND METHODS

Animal intervention was based on glycine levels which was found to be mainly elevated in cataract monkeys compared to non-cataract especially in CSF. Twelve individuals were assigned into three groups (control, induced and spontaneous) consisting of four animals. As outlined in chapter two, the animal intervention consisted of phase one (valproate induction) and phase two (treatment) (Table 3.1). Valproate was administered to four non-cataract animals to induce hyperglycinemia, thereafter, both induced and spontaneous groups were treated with sodium benzoate and dextromethorphan to normalise glycine levels in CSF and plasma. Routine screening for glycine levels, haematology and biochemical parameters was performed to determine the effect of valproate, sodium benzoate and dextromethorphan. Significant changes in the induced and spontaneous group were compared to the control group.

Table 3.1: Summary of animal intervention

PHASE: 1				
Groups	**No. of Subjects**	**Treatment**	**Daily Dose (mg/kg bwt)**	**Duration**
Group 1 Control	4	Maintenance diet	None	3 weeks
Group 2: Induced	4	Valproate	50 mg/kg/day	
Blood (2 ml), 0.5 ml CSF and urine was collected at baseline. To monitor glycine level, blood and urine sampling was performed every week except for CSF which was collected at the end of week three valproate induction.				
PHASE: 2				
Group 1 Control	4	Controls	Maintenance diet	4 weeks
Group 2 Induced	4	Sodium benzoate and Dextromethorphan	250mg/kg/day 5mg/kg/day	
Group 3 Spontaneous	4	Sodium benzoate and Dextromethorphan	250mg/kg/day 5mg/kg/day	
Blood and urine was collected every week of the treatment to monitor the efficacy of the compounds, whereas CSF was only collected at the end of the treatment and washout period.				

3.3 RESULTS

3.3.1 The analysis of biochemical and haematological parameters

During the animal intervention, no signs or symptoms that were indicative of unwell-being or distress were observed throughout the study, as defined in the observation logs (Appendix A2). The biochemical (Table 3.2) and haematological (Table 3.3) analysis showed a significant difference for magnesium (P=0.0017), LDL-C (P = 0.04), urea (P = 0.02) and MCH (0.02) between the control, induced and spontaneous group. The differences between these groups were observed during the treatment period for magnesium (Figure 3.1) and LDL-C (Figure 3.2) whereas urea (Figure 3.3) and MCH (Figure 3.4) changes were at baseline. Although the remaining biochemical and haematological parameters were not significant (Appendix A,

Table 1A), group specific changes were noted. Further analysis showed that valproate had an impact on the levels of ALP, phosphate and platelet count. The levels of ALP were elevated during the induction and treatment phase (Figure 3.5). Additionally, phosphate levels (Figure 3.6) increased by 54% from baseline to induction period and the treatment reduced phosphate levels by 31%. Similarly, platelet count (Figure 3.7) indicated a 30% increase from baseline to induction phase and was reduced by 12% after treatment.

Table 3.2: Summary of biochemistry observation

Variable	**Significance between groups**	**Interpretation (*P*<0.05)**	**Figure**
ALT	No		
AST	No		
GGT	No		
Mg	Yes (P = 0.0017)	Control vs Group 2 = 0.02 Group 2 vs Group 3= 0.0017	3.1
Total Proteins	No		
Total Bilirubin	No		
Direct Bilirubin	No		
Albumin	No		
Globulin	No		
HDL-C	No		
LDL-C	Yes (P = 0.04)	Group 2 vs Group 3= 0.04	3.2
Urea	Yes (P = 0.02)	Control vs Group 2 = 0.03 Group 2 vs Group 3= 0.04	3.3
Creatinine	No		
Triglycerides	No		
ALP	No	Period specific for group 2	3.5
Ca	No		
Na	No		
Cl	No		
Anionic gap	No		
P	No	Period specific for group 2	3.6
K	No		
CO_2	No		
CK	No		

Table 3.3: Summary of haematological observation

Variable	Significant	Interpretation (*P*<0.05)	Figure
RBC	No		
WBC	No		
MCV	No		
MCH	Yes (P = 0.02)	Group 2 vs Group 3= 0.02	3.4
MCHC	No		
RDW	No		
Hb	Yes		
Neutrophils	No		
Basophils	No		
Lymphocytes	No		
Monocytes	No		
Platelets	No	Period specific for group 2	3.7
Hct	No		

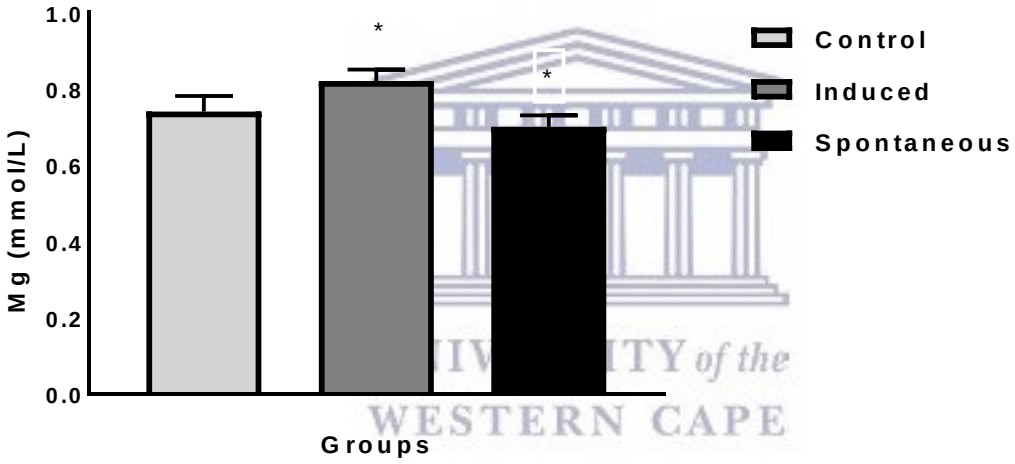


Figure 3.1: Magnesium (Mg). The control group received a maintenance diet throughout the intervention study. The induced group received valproate at 50mg/kg/day for three weeks and both group two and three (spontaneous) were treated with sodium benzoate at 250mg/kg/day and dextromethorphan (5mg/kg/day) for four weeks. * represent significant difference (p<0.05)

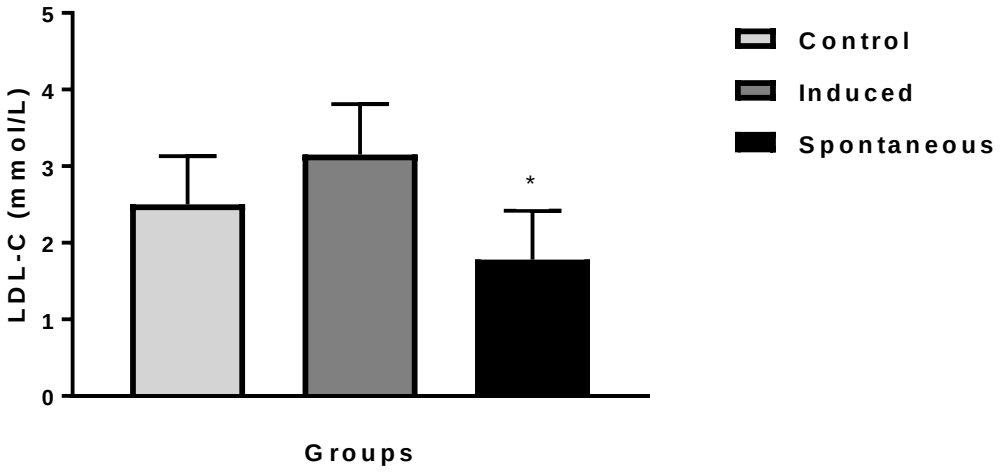


Figure 3.2: Low density lipoprotein cholesterol (LDL-C). The control group received a maintenance diet throughout the intervention study. The induced group received valproate at 50mg/kg/day for three weeks and both group two and three (spontaneous) were treated with sodium benzoate at 250mg/kg/day and dextromethorphan (5mg/kg/day) for four weeks. * represent significant difference ($p<0.05$)

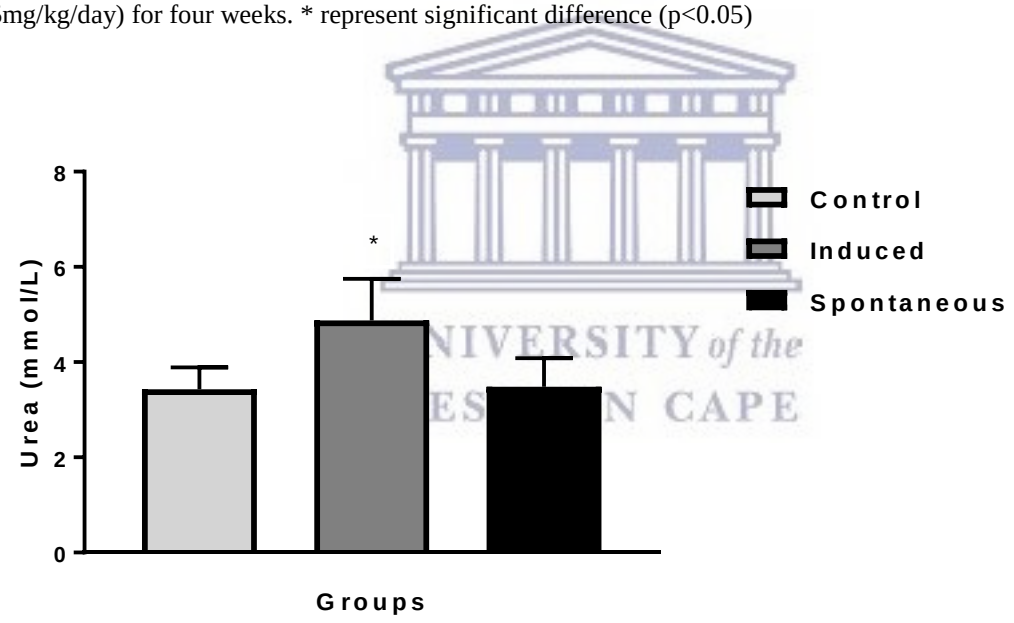


Figure 3.3: Urea. The control group received a maintenance diet throughout the intervention study. The induced group received valproate at 50mg/kg/day for three weeks and both group two and three (spontaneous) were treated with sodium benzoate at 250mg/kg/day and dextromethorphan (5mg/kg/day) for four weeks. * represent significant difference ($p<0.05$)

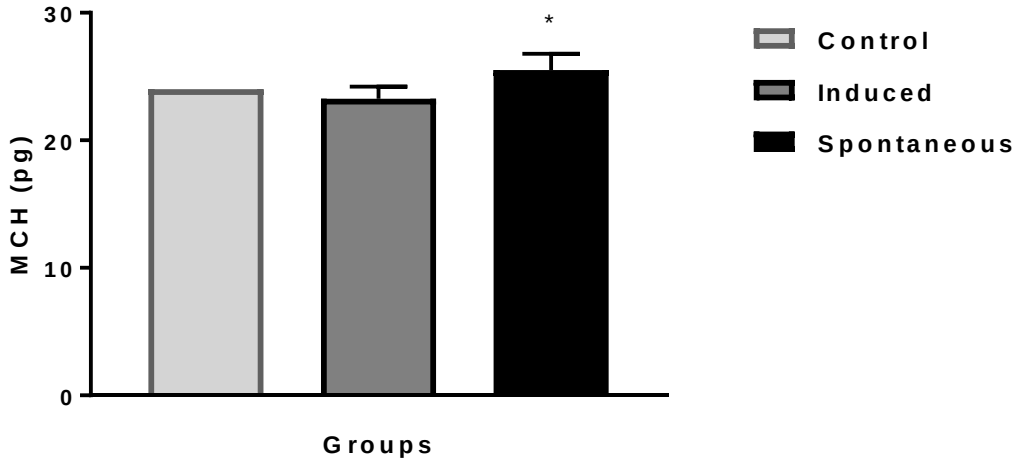


Figure 3.4: Mean Corpuscular Haemoglobin (MCH). The control group received a maintenance diet throughout the intervention study. The induced group received valproate at 50mg/kg/day for three weeks and both group two and three (spontaneous) were treated with sodium benzoate at 250mg/kg/day and dextromethorphan (5mg/kg/day) for four weeks. * represent significant difference ($p<0.05$)

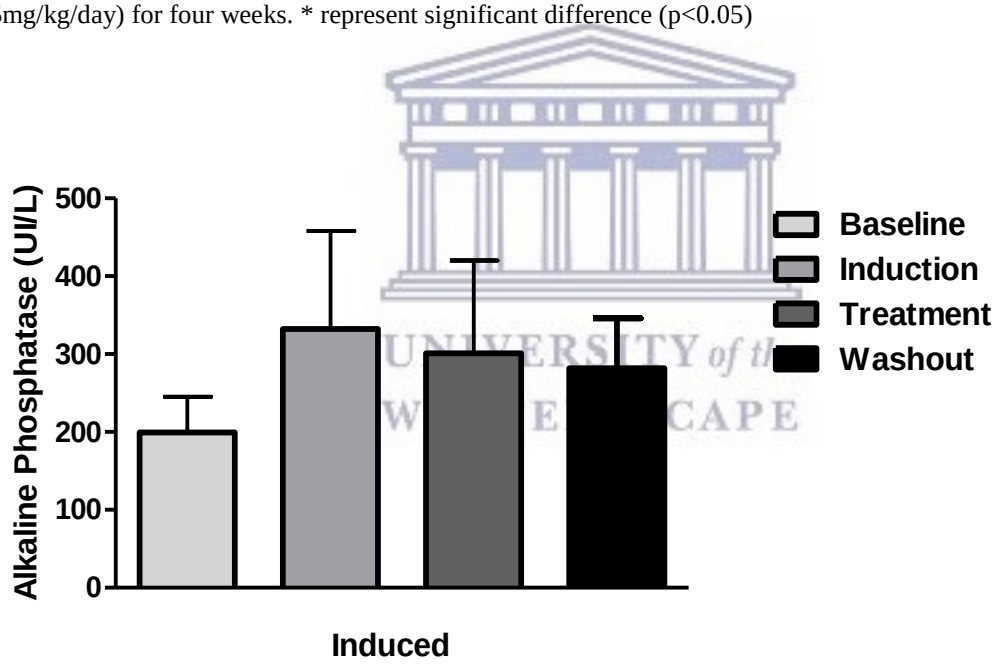


Figure 3.5: Alkaline phosphatase (ALP). Group two induced with valproate at 50mg/kg/day for three weeks and treated with sodium benzoate at 250mg/kg/day and 5mg/kg/day dextromethorphan for four weeks. Washout period was four weeks.

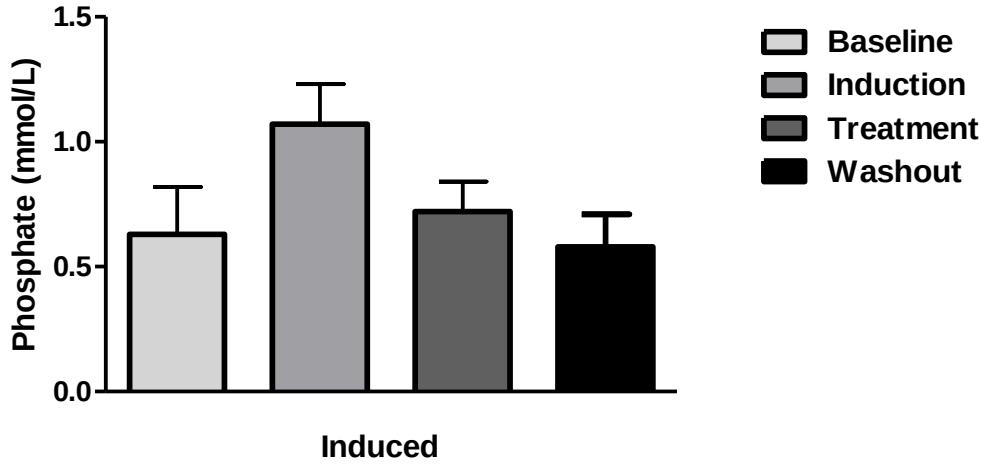


Figure 3.6: Phosphate. Group two induced with valproate at 50mg/kg/day for three weeks and treated with sodium benzoate at 250mg/kg/day and 5mg/kg/day dextromethorphan for four weeks. Washout period was four weeks.

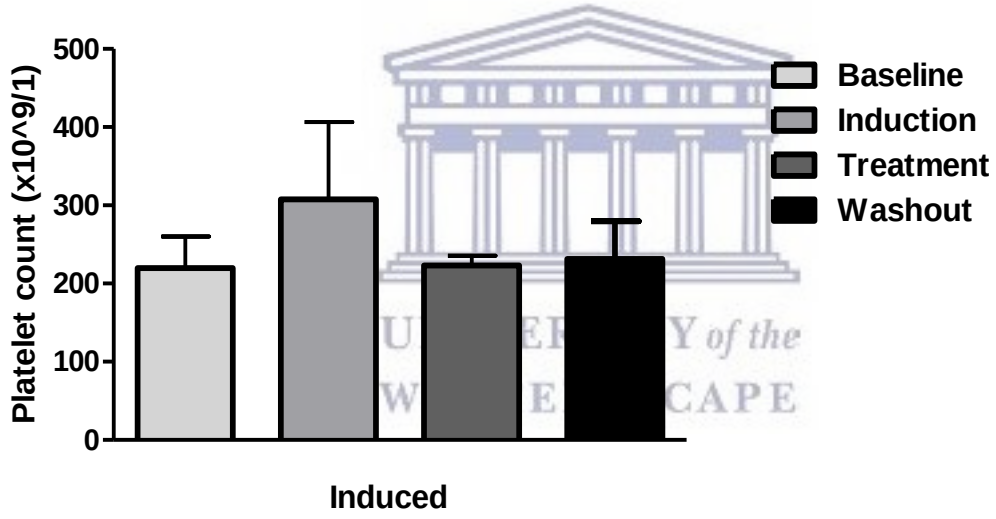


Figure 3.7: Platelet count. Group two induced with valproate at 50mg/kg/day for three weeks and treated with sodium benzoate at 250mg/kg/day and 5mg/kg/day dextromethorphan for four weeks. Washout period was four weeks.

3.3.2 Effect of valproate, sodium benzoate and dextromethorphan in glycine levels

Glycine analysis showed a significant difference in CSF ($P<0.001$) and plasma levels ($P<0.002$) between the groups. The spontaneous group had high baseline and washout glycine levels in CSF compared to the control group (Figure 3.8, Appendix A, Table A6). The glycine levels in plasma for both experimental groups were different from the control during the treatment period (Figure 3.9). Additionally, the spontaneous group showed a significant difference from baseline to treatment for CSF ($p<0.04$) and plasma ($p<0.007$) (Figure 3.10). Therefore, the treatment was effective in normalising glycine levels in CSF and plasma.

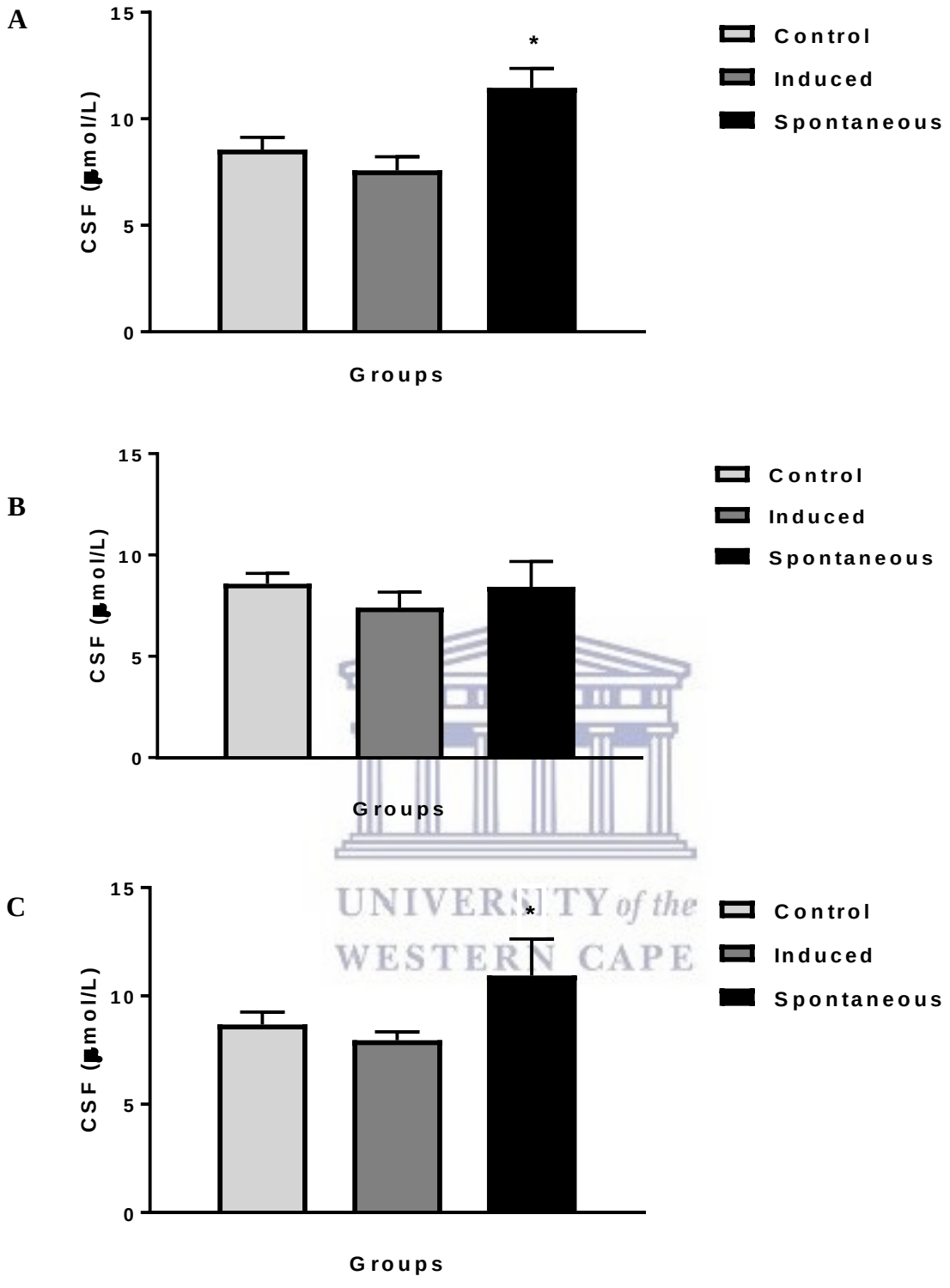


Figure 3.8: Glycine in CSF of vervet monkeys. Results for control, induced and spontaneous groups: (A) Baseline, (B) Treatment and (C) Washout period. The control group received a maintenance diet throughout the intervention study. The induced group received valproate at 50mg/kg/day for three weeks and both group two and three (spontaneous) were treated with sodium benzoate at 250mg/kg/day and dextromethorphan (5mg/kg/day) for four weeks. * represent significant difference ($p<0.05$)

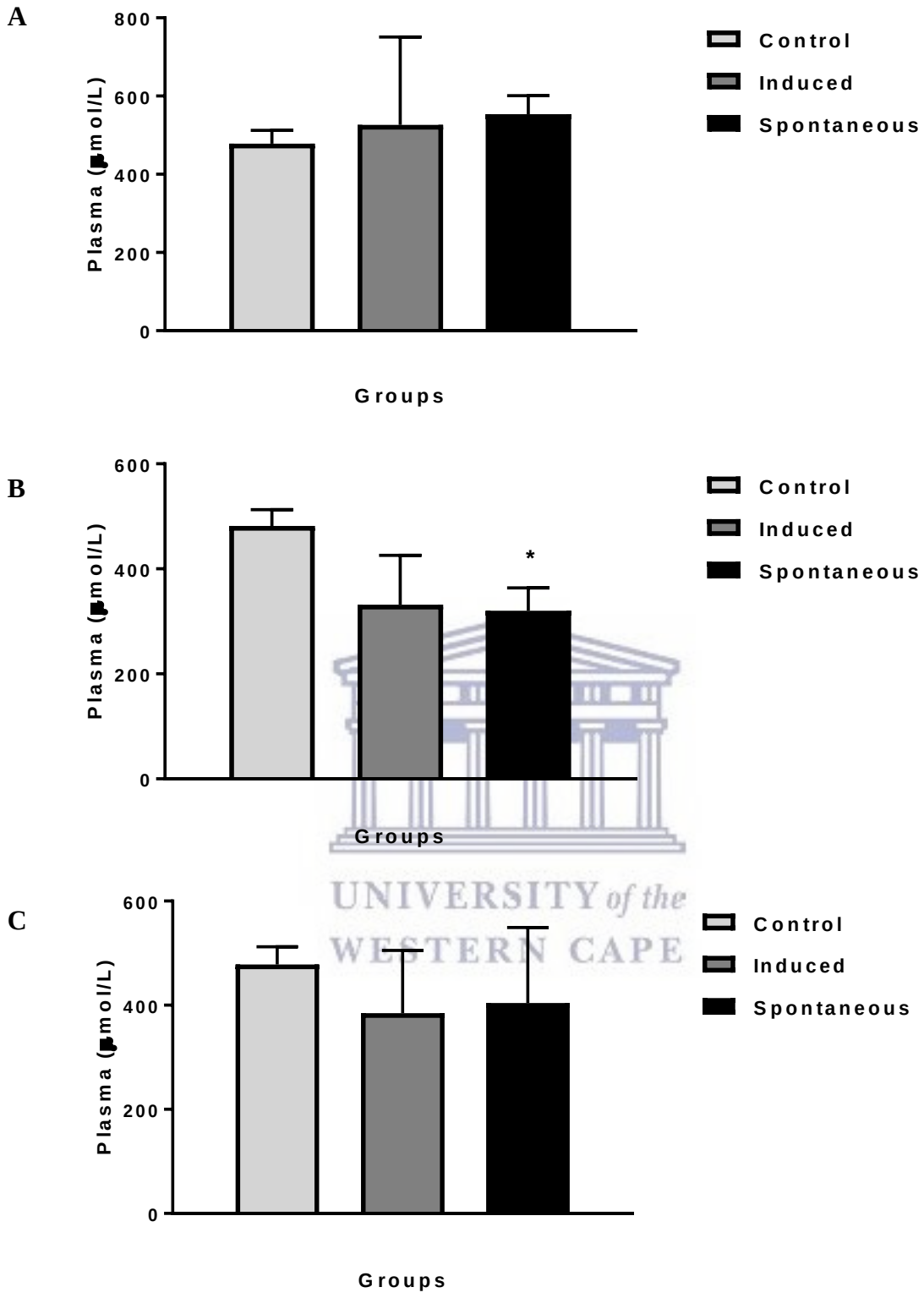


Figure 3.9: Glycine in plasma for vervet monkeys. Results for control, induced and spontaneous groups: (A) Baseline, (B) Treatment and (C) Washout period. The control group received a maintenance diet throughout the intervention study. Valproate was administered at 50mg/kg/day in group two for three weeks and both group two and three were treated with sodium benzoate at 250mg/kg/day for four weeks. * represent significant difference ($p<0.05$).

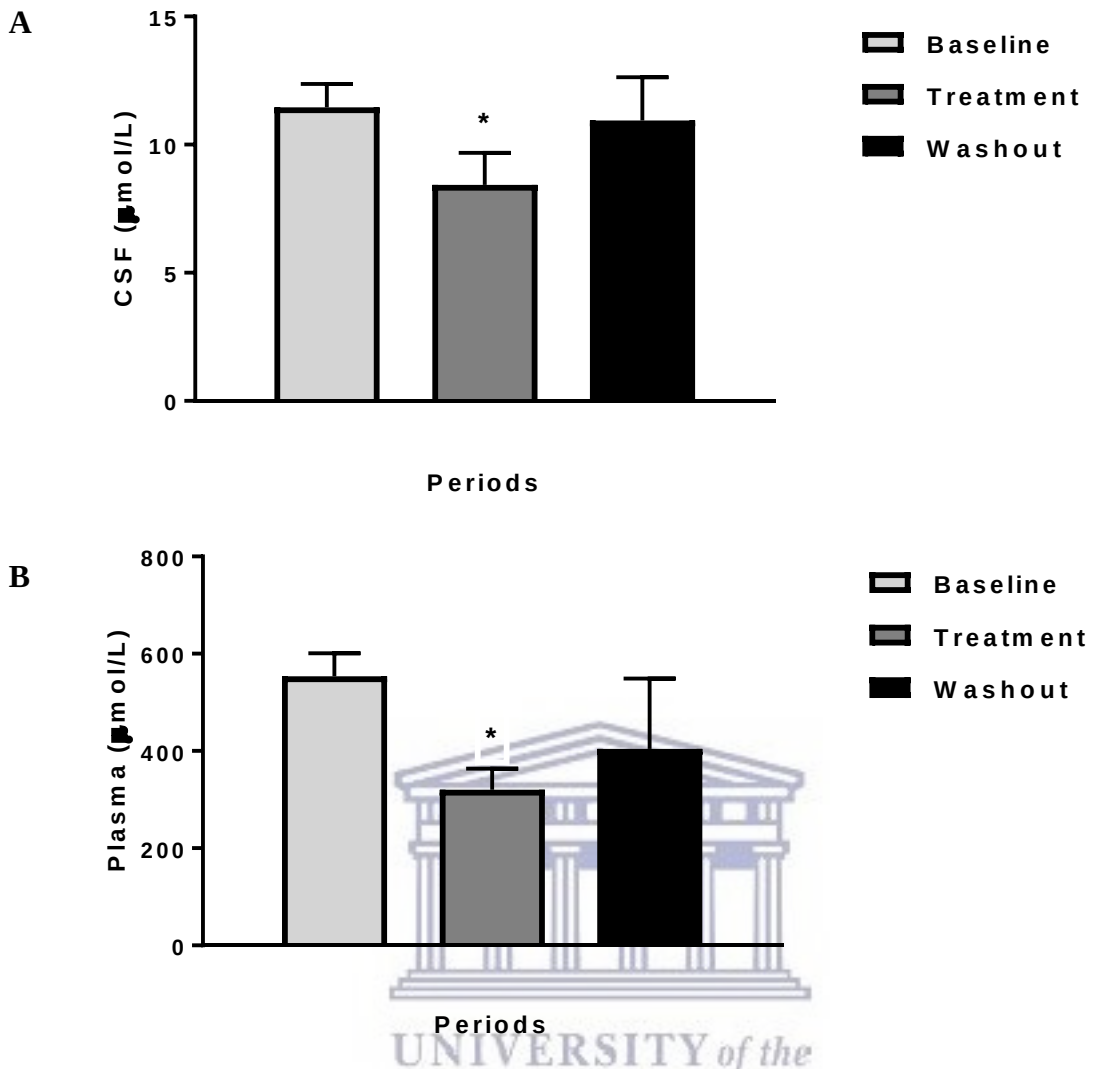


Figure 3.10: Group three (spontaneous) glycine levels in vervet monkeys. (**A**) CSF and (**B**) Plasma. Group three was treated with sodium benzoate at 250mg/kg/day and dextromethorphan (5mg/kg) for four weeks. * represent significant difference ($p<0.05$)

3.4 DISCUSSION

Valproate has been used to treat epilepsy and different types of seizures (Aldenkamp et al., 2006, Ben-Menachem et al., 2006). Valproate is known to cause hepatotoxicity and is associated with serious adverse reactions such as hyperammonemia and thrombocytopenia (Star et al., 2014, Koenig et al., 2006). These adverse reactions occur due to inborn error in valproate metabolism, its reactive metabolites and inhibition of beta-oxidation (Fromenty and Pessayre, 1995). Additionally, underlying genetic or acquired mitochondrial abnormalities are reported to contribute to valproate hepatotoxicity (Boelsterli and Lim, 2007).

In this study, the efficacy of valproate induction (50 mg/kg), sodium benzoate (250 mg/kg) and dextromethorphan (5 mg/kg) treatment was evaluated for the period of 13 weeks. The observed biochemical and haematological changes were mainly due to valproate induction (50 mg/kg), however, no obvious signs of illness such as hepatotoxicity or hyperammonemia were detected in this group. In phase one, elevated glycine levels in CSF and plasma of one animal were observed after valproate induction, while the rest of the group showed varying results. Inter-individual variations has been reported in human patients on valproate therapy, thereby resulting in insufficient treatment outcomes (Chatzistefanidis et al., 2012). Therefore, it is possible that the observed variations in these monkeys contributed to the inconclusive findings especially in the monkeys that did not respond to induction in phase one.

Moreover, the inconclusive findings to induce hyperglycinemia can also be attributed to the short treatment duration and the dose that was chosen based on previous studies in vervet monkeys (Viljoen et al., 2012). Nevertheless, valproate findings confirmed that just like in humans, vervet individual monkeys also show variations thus influencing the effectiveness of the induction.

The biochemical and haematological parameters showed significant variations between the treated groups and the control group for Mg (P=0.0017), LDL-C (P = 0.04), urea (P = 0.02) and MCH (0.02). Most of these changes were attributed to the induced group, which was treated with valproate. Based on literature review, valproate was expected to cause changes in the biochemical parameters especially for kidney and liver biomarkers, hence, a small dose was chosen to induce hyperglycinemia over a short period of time. Valproate is known to increase ALP which is considered a marker of hepatocellular strength since its origin is from the liver and bone (Tolou-Ghamari et al., 2013). An increase in ALP levels without elevated GGT levels means that valproate caused an impact in the liver (Ahmed and Siddiqi, 2006). Although there were no significant clinical signs of liver damage in this study, valproate has a potential in damaging the liver in vervet individuals. A slight decrease in ALT and AST was also observed however according to Chand and co-workers (Chand et al., 2011), this change does not trigger liver disease while minor elevations in aminotransferases are the first signs of hepatoxicity (Karaoglu et al., 2009).

Similar to this study, a decrease in ALT in rats (Espandiari et al., 2008) and increased ALP levels in epileptic human patients treated with valproate have been reported (Hussein et al., 2013). However, temporary elevation of ALT and AST in epileptic human patients (Felker et al., 2003) and in pregnant mice following valproate administration were also observed (Amrani et al., 2013). Therefore, valproate administration in humans, NHPs and rodents is capable of interfering with aminotransferases, even though the changes were not significant in this study.

In this study, valproate slightly elevated platelet counts and this response has not been reported in association with valproate therapy in humans. Most studies have indicated that valproate causes thrombocytopenia (Hauser et al., 1996, Espandiari et al., 2008, Nasreddine and

Beydoun, 2008) which normally occurs several months after valproate therapy. The degree of thrombocytopenia correlates with the levels of valproate (Delgado et al., 1994, Karaoglu et al., 2009) and is very common in pediatric patients with an incident of 5-40% (Karaoglu et al., 2009).

Nevertheless, the vervet reference values for haematology and biochemical parameters were used to determine whether the changes obtained in this study could be disease-causing. The comparison to reference values showed that phosphate (0.63± 0.19, reference 1.30 ± 0.29 mmol/L) (Figure 3.6) and platelet count (199.25± 46.00, reference 317.85± 50.89 x10^9/1) (Figure 3.7) were decreased but within the normal levels at baseline. The low levels showed that there are environmental factors such as age, sex and captivity conditions (Castro et al., 2015) that also contribute to changes in haematological and biochemical factors as these monkeys were not under any treatment prior this study. Therefore, valproate increased phosphate and platelet count in the induced group but the changes were reversed by the treatment.

With regards to glycine analysis, the spontaneous group showed elevated CSF and plasma compared to the control and induced group. This observation indicated that there might be an association between cataract and hyperglycinemia however, an association test is required to confirm the findings. Interestingly, the treatment normalised glycine levels in the spontaneous group (Figure 3.10), while it was less effective in normalising CSF levels in the induced group and this can be due to the dose that was chosen. The dose might have been inadequate for the induced group, therefore, a higher dose can be considered. A similar observation has been reported in neonates where CSF levels were not normalised by the treatment (Hamosh et al., 1998, Kojima-ishii et al., 2008, Madu and Oliver, 2013). Therefore, treatment with sodium

benzoate and dextromethorphan was found to be more effective in reducing glycine levels in the spontaneous group of vervet monkeys.

3.5 CONCLUSION

The findings of this study demonstrated that low dose (50 mg/kg) of valproate did not have any significant clinical impact on liver, kidney and haematological parameters even though changes were observed for ALP, phosphate and platelet count. Individual variations were observed in glycine levels (CSF and plasma) in the induced group. The findings suggested that the variations might have been of genetic origin, thus affecting valproate metabolism. Therefore, these findings do not bring into disregard the efficacy of valproate but further studies using a higher dose, larger group size and extended induction period are suggested. In conclusion, sodium benzoate and dextromethorphan treatment normalised glycine levels in hyperglycinemic vervet monkeys. The subsequent chapter will correlate the effect of valproate with genotyping and gene expression of selected genes that are known to play a role in valproate glucuronidation in humans.

CHAPTER FOUR:

Association of valproate and sequence variants in *UGT1A6* and *UGT1A9* using a colony of captive-bred vervet monkeys

4.1 INTRODUCTION

There is a growing interest in biochemical and clinical studies, to identify genetic polymorphisms using a combination of molecular sequencing technology (Evans and Johnson, 2001). In general, sequencing approaches serves as a foundation to determine an appropriate dose for each individual based on their genetic make-up. As a result, genetic polymorphisms have been identified in genes or proteins encoded for drug metabolizing enzymes, drug transporters and drug receptors (Krishnaswamy et al., 2005, Evans and Johnson, 2001). These polymorphisms are known to affect pharmacokinetics and pharmacodynamics of drug efficacy, toxicity and influence the way individuals respond to certain medication (Salari et al., 2012). Thus, genetic factors that have an influence on gene function, associated proteins and other environmental factors must be taken into consideration when determining an effective dose.

Glucuronidation is an important metabolic pathway for several antiepileptic drugs (AEDs) (Argikar and Remmel, 2009). These AEDs including valproate are excreted extensively as their glucuronides (Levy et al., 2002). This pathway occurs through uridine diphosphate glucuronosyltransferase (UGT) which has many isoforms that are involved in metabolism of various therapeutic compounds. In humans, the glucuronidation of valproate, also known as valproic acid (VPA) is reported to be carried out by UGTs such as *UGT1A3*, *UGT1A6*, *UGT1A9*, *UGT2B7*, and *UGT2B15* (Green et al., 1998, Green and Tephly, 1996, Ethell et al., 2003), however, *UGT1A6* and *UGT1A9* account for major valproate clearance. Valproate is

directly glucuronidated to form ester (acyl) glucuronide that is excreted in the urine, accounting for 30 –70% of the administered dose (Dickinson et al., 1989).

A variety of sequence variants, especially single nucleotide polymorphisms (SNPs) in exon or intron and promoter region has been reported in human UGTs (Guillemette, 2003). Such variants can influence drug metabolism and excretion (Saruwatari et al., 2010) resulting in inter-individual variability (Guillemette, 2003). Additionally, valproate is known to be affected by sequence variants thus resulting in inter-individual variability in pharmacokinetics and pharmacodynamics (Jain et al., 2015). In the previous chapter, the use of valproate for hyperglycinemia induction was discussed, but preliminary findings were inconclusive in inducing hyperglycinemia hence *UGT1A6* and *UGT1A9* genes were prioritized in this chapter. It has been reported that mutations or polymorphisms in *UGT1A6* and *UGT1A9* genes affect valproate therapy (Aphichartphunkawee et al., 2014). These two UGT genes are known to transform small lipophilic molecules (steroids, bilirubin, hormones, and drugs) into water-soluble compounds so that they can be excreted in urine (Ismail et al., 2010). Both genes code for functional protein (Girard et al., 2004) and play an important role in valproate therapy (Court et al., 2004, Johannessen, 2000, Sun et al., 2007).

Thus far, three genetic variations (S7A, T181A and R184S) in *UGT1A6* have been reported in humans (Jain et al., 2015, Krishnaswamy et al., 2005, Munisamy et al., 2013). These variants have been associated with valproate therapy in epileptic patients. Carriers of these polymorphisms tend to require more dosage than the wild type (Hung et al., 2011) and have slower metabolism of some phenolic substrate (Ciotti et al., 1997).

Conversely to *UGT1A6*, polymorphic expression and variable levels of glucuronidation activity of *UGT1A9* protein has been reported in the promoter region and exon one (Girard et al., 2004, Yamanaka et al., 2004). Two polymorphisms located in the promoter region (-275 T>A and -2152 C>T) of *UGT1A9* are associated with increased expression in the liver (Girard et al., 2004). Additionally, inter-individual variation due to sequence variants has been reported (Kuypers et al., 2005), thus influencing the function and expression level of *UGT1A9* (Girard et al., 2004). Therefore, identification of sequence variants or SNPs in vervet *UGT1A6* and *UGT1A9* will contribute in understanding the inter-individual variations that was observed in the valproate induced group and confirm the role that these variants have on the metabolism of valproate.

The purpose of this chapter was to identify sequence variants in *UGT1A6* and *UGT1A9* and to correlate it with gene expression. Since these monkeys were induced with valproate, the impact of the compound will also be evaluated in the induced group.

4.2 MATERIALS AND METHODS

For the purpose of this chapter, four non-cataract individuals that were induced with valproate in chapter three were selected. The control group consisting of four healthy monkeys was included as a negative control for sequencing and gene expression. The sequences for *UGT1A6* and *UGT1A9* were retrieved in public domain as mentioned in chapter two. Primers were designed for the unique exon 1 as well as for the common exon 2-5. The designed primers (Appendix B3) were amplified using standard PCR and sequenced to identify sequence variants. The impact of valproate, sodium benzoate and dextromethorphan on gene expression was also determined using qRT-PCR as described in chapter two. Two housekeeping genes

(*PGK1* and *GAPDH*) (Table 2.8) were used to normalise mRNA levels of *UGT1A6*, *UGT1A9*, *GLDC* and *AMT*. The findings were further normalised using the control group as a calibrator.

4.3 RESULTS

4.3.1 Identification of sequence variants in *UGT1A6* and *UGT1A9*.

The correct size of PCR amplicons of *UGT1A6* and *UGT1A9* genes were as expected when subjected to 2% agarose gel electrophoresis (Figure 4.1). Sequencing of the coding region of *UGT1A6* revealed four heterozygous missense (R6H, R254Q, M298I and A279T) (Figure 4.2), three silent mutations (S12S, G26G, V48V) and four variants that were unique to vervet monkeys (S7A, V15I, D51A, and T181A) (Table 4.1, Figure 4.3) in exon one. The *UGT1A9* sequencing analysis revealed two transition silent mutations (Q88Q and F197F) and 12 sequence variants (M59I, D85V, K132M, Y189H, I193V, R208W, I211V, M212T, L219F, R222Y, A227I and P238S) (Figure 4.4) in exon one. Silent mutations were identified in the common regions of exon three (N347N, T348T, N357N) and five (F517F) of both genes (Table 4.1). The sequence variants were located in conserved regions when aligned with rhesus and human sequences except for the change in codon 517, which was different between human (L517F) and rhesus (F517F) (Figure 4.3).

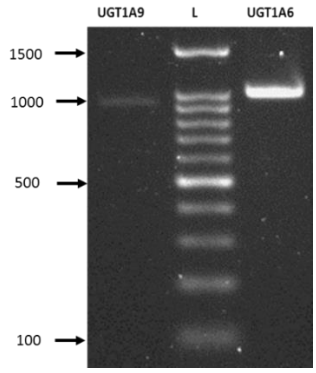


Figure 4.1: Genetic analysis of selected captive- bred vervet monkeys in group two. A 2% agarose gel electrophoresis displaying exon one of *UGT1A9* (879bp) and *UGT1A6* (994bp) genes. L = 100-1500bp DNA ladder.

Table 4.1: Summary of sequence variants in *UGT1A6* and *UGT1A9* of selected vervet monkeys

Gene	Exon	Nucleotide change	Type of change	Amino acid change	Type of mutation	Monkey ID	Affected controls
UGT1A6	1	G17>A	Transition	R6H	Missense	2	2
		C36>T	Transition	S12S	Silent	2	2
		C79>T	Transition	G26G	Silent	2	2
		T143>C	Transition	V48V	Silent	2	2
		G623>A	Transition	R254Q	Missense	2	2
		G697>A	Transition	A279T	Missense	3	2
		T756>G	Transversion	M298I	Missense	2	2
	3	T980>C	Transition	N347N	Silent	1	0
		G983>A	Transition	T348T	Silent	2	0
		C1010>T	Transition	N357N	Silent	3	0
	5	C1490>T	Transition	F517F	Silent/miss	1	0
UGT1A9	1	G301>A	Transition	Q88Q	Silent	1	0
		C628>T	Transition	F197F	Silent	1	0
	3	T1035>C	Transition	N346N	Silent	1	0
		G1038>A	Transition	T347T	Silent	2	0
		C1065>T	Transition	N356N	Silent	3	0
	5	C1545>T	Transition	F516F	Silent/miss	1	0

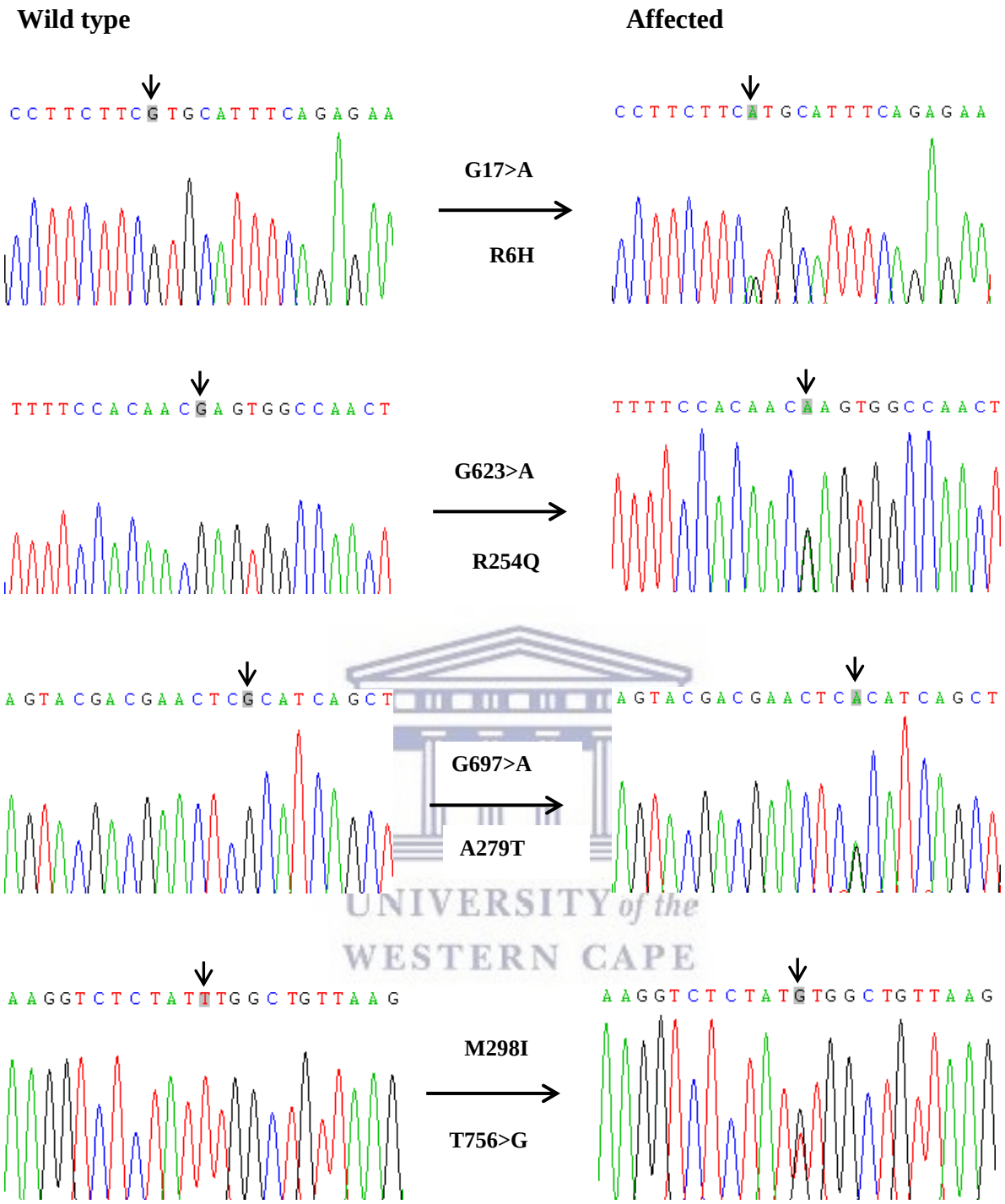


Figure 4.2: Genetic analysis of selected group two vervet monkeys. Sequence chromatogram displaying four heterozygous sequence variants (G17>A, G623>A, G697>A and T756>G) located in exon one of *UGT1A6* resulting in missense mutations (R6H, R254Q, A279T and M298I) respectively. The arrows on the chromatographs indicate the position of each sequence variant.

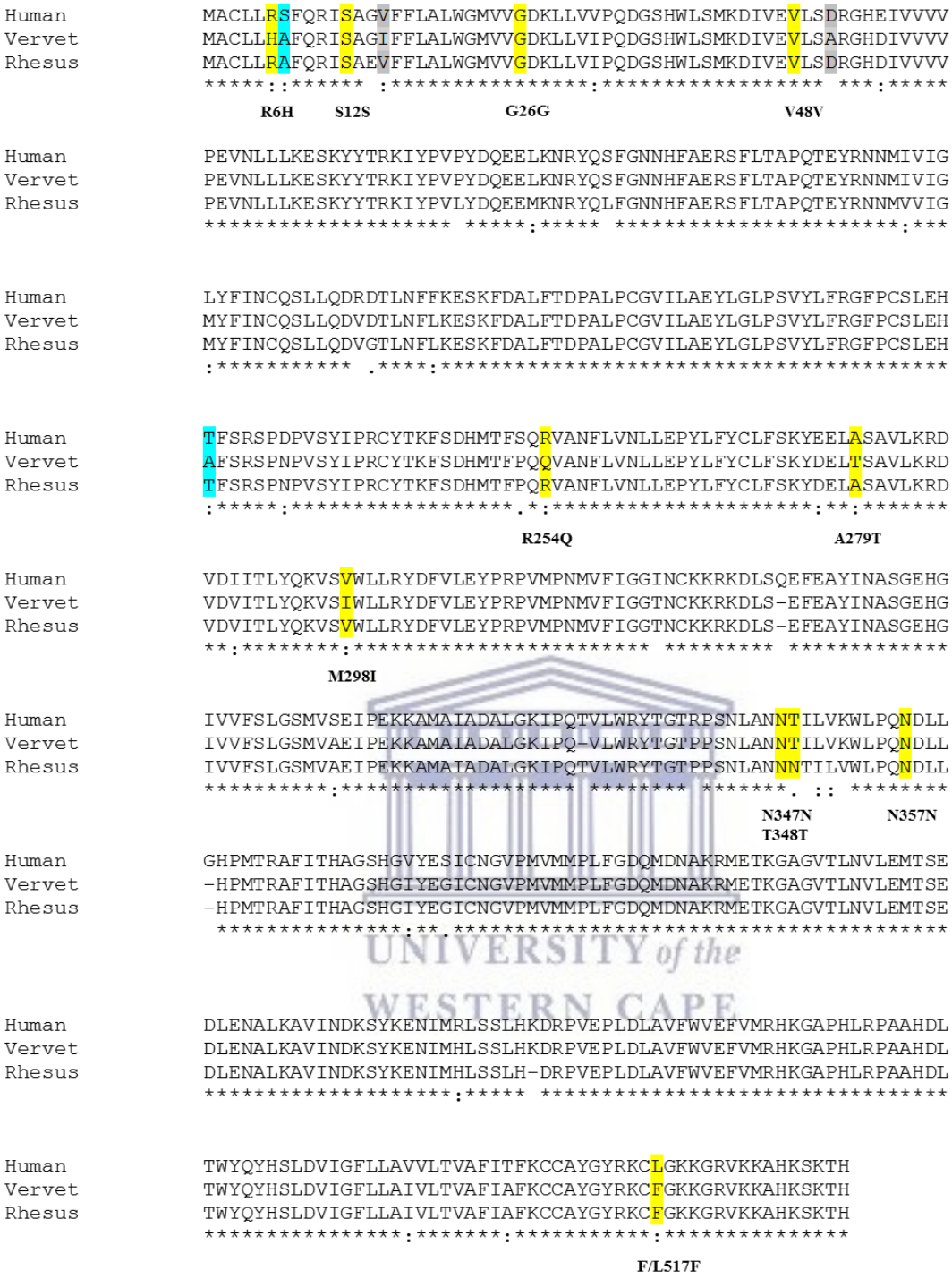


Figure 4.3: *UGT1A6* mRNA sequence alignment between human, vervet and rhesus species using ClustalW. The sequence highlighted yellow represent the missense sequence changes in *UGT1A6* (Table 1), the sequence highlighted grey represent sequence changes observed in all the vervet monkeys in this study and the sequence highlighted blue represents sequence changes in vervet monkeys previously reported in human studies. Below the protein sequences is a key denoting conserved sequence (*), conservative mutations (:), semi-conservative mutations (.), and non-conservative mutations ( ).

Figure 4.4: *UGT1A9* mRNA sequence alignment between human, vervet and rhesus species using ClustalW. The sequence highlighted yellow represent the missense and silent sequence changes in *UGT1A9* (Table 1), the sequence highlighted grey demonstrates unique variants observed in the vervet species compared to human and rhesus sequences. Below the protein sequences is a key denoting conserved sequence (*), conservative mutations (:), semi-conservative mutations (.), and non-conservative mutations ( ).

4.3.2 Gene expression analysis

4.3.2.1 RNA integrity and purity

RNA integrity was confirmed using Agilent 2100 bioanalyzer (Table 4.2, Figure 4.5A and B, Appendix B10). During the induction phase, only the induced group was analysed. Furthermore, the PCR with ActB primers (Appendix B, Table B1) confirmed that the RNA was not contaminated with genomic DNA.

Table 4.2: Extracted RNA samples with PAXgene Blood RNA Kit

Group	Animal ID	Baseline			Induction			Treatment			Washout		
		Conc. (ng/µl)	260/280	260/230	Conc. (ng/µl)	260/280	260/230	Conc. (ng/µl)	260/280	260/230	Conc. (ng/µl)	260/280	260/230
1.Control	127	65.9	2.11	1.35				61.6	2.06	1.86	79.6	2.08	1.77
	145	72.3	2.06	1.38				57.9	2.05	1.54	58.9	1.99	1.57
	178	127.4	2.07	2.31				54.2	2.07	0.82	54.7	2.01	1.2
	399	***3.86**	**2.44**	**1.35**				7.4	1.98	0.75	56.9	2.02	2.05
2.Induced	104	71.8	2.07	0.95	60.2	2.1	1.68	54.4	2.02	2.02	68.6	2.02	1.93
	113	117.6	2.09	1.92	65.9	2.1	1.69	78.5	2.03	1.94	122	2.05	1.98
	338	52.8	2.06	1.86	44.4	2.06	1.57	39.5	2.06	1.86	33	2.01	0.92
	438	74.6	2.09	0.75	75.2	2.1	1.84	76.5	2.06	1.89	74.2	2.04	1.91
3. Spontaneous	**398**	***4.0**	**-**	**-**				67.6	2.1	0.82	123.5	2.06	1.83
	409	87.6	2.08	1.92				76.7	2.08	1.84	88.3	2.07	0.98
	412	***2.1**	**-**	**-**				82	2.08	1.91	83.6	2.06	2.05
	416	52.7	2.04	1.30				73.8	2.07	1.35	47.6	2.03	1.78

(*): Samples in bold had low concentration for baseline RNA extraction.

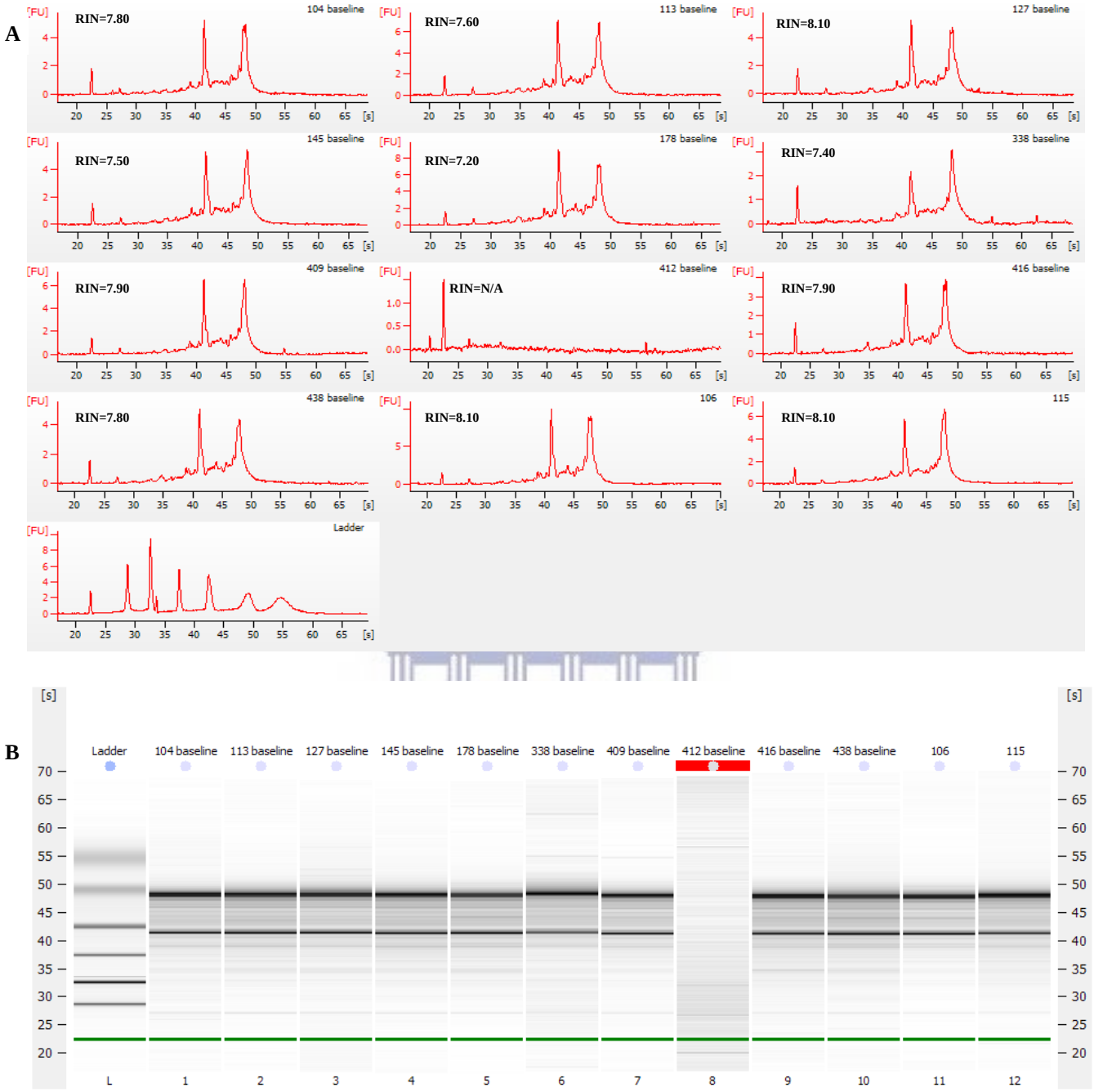


Figure 4.5: mRNA Bioanalyzer analysis. (**A**) Electropherograms of baseline total RNA samples. (**B**) Gel showing total RNA gel like-image for baseline samples (412 sample was degraded).

4.3.2.2 UGT1A6 and *UGT1A9 gene expression*

The *UGT1A6* gene expression was only detected in baseline samples, while *UGT1A9* gene expression showed a slight increase during the induction and treatment phase (Figure 4.6), however this change was not statistically significant. Gene expression of *UGT1A9* differed between individual monkeys M104 and M113, the latter indicated higher expression than M104 at baseline (Figure 4.7).

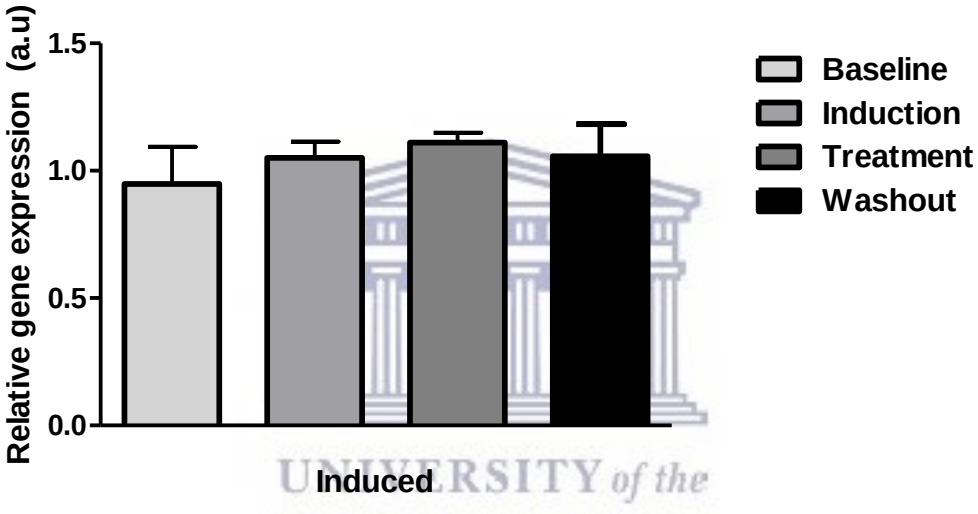


Figure 4.6: *UGT1A9* mRNA gene expression in vervet monkeys. The induced group received 50mg/kg/day of valproate and treated with sodium benzoate (250mg/kg/day) and dextromethorphan (5mg/kg/day) together with the spontaneous group. The data was expressed as mean ± SD and mRNA expression in a.u. (arbitrary units).

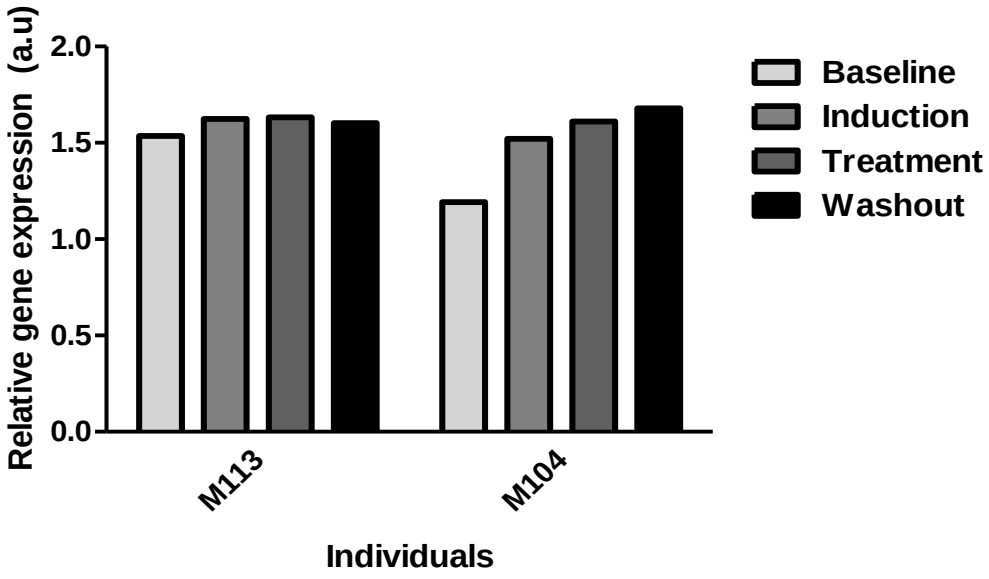


Figure 4.7: Comparison of *UGT1A9* mRNA gene expression in wild type (M113) and mutated (104) vervet monkeys. The induced group received 50mg/kg/day of valproate and treated with sodium benzoate (250mg/kg/day) and dextromethorphan (5mg/kg/day) together with the spontaneous group. The data was expressed as mean ± SD and mRNA expression in a.u. (arbitrary units).

4.3.2.3 GLDC and *AMT gene expression*

The impact of valproate induction was determined for *GLDC* and *AMT* in the induced group from baseline to washout. Gene expression findings for *GLDC* (Figure 4.8A) were not significant compared to the control although there was a 18.5% decrease during the induction phase in the induced group (Figure 4.8B). Conversely, *AMT* (Figure 4.9A) was higher than the control group at baseline, however, valproate significantly decreased the expression levels of this gene during the induction and washout period (Figure 4.9B).

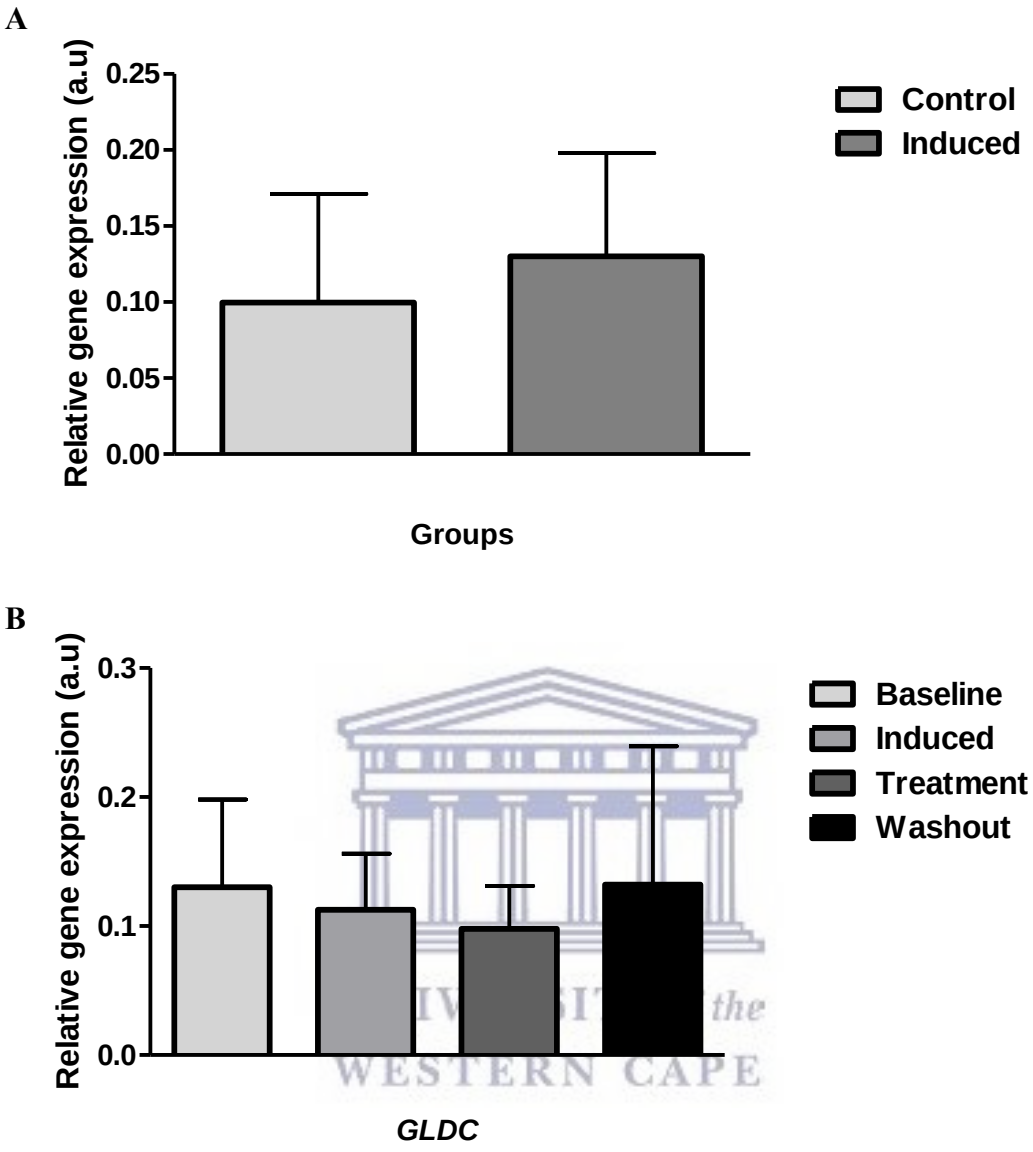


Figure 4.8: ***GLDC*** **mRNA gene expression. A)** The expression of control compared to the induced group. The control group received a maintenance diet throughout the study while **t**he induced group received 50mg/kg/day of valproate and treated with sodium benzoate (250mg/kg/day) and dextromethorphan (5mg/kg/day) together with the spontaneous group. **B**) Expression of *GLDC* at baseline, treatment followed by washout period of four weeks. The data was expressed as mean ± SD and mRNA expression in a.u. (arbitrary units).

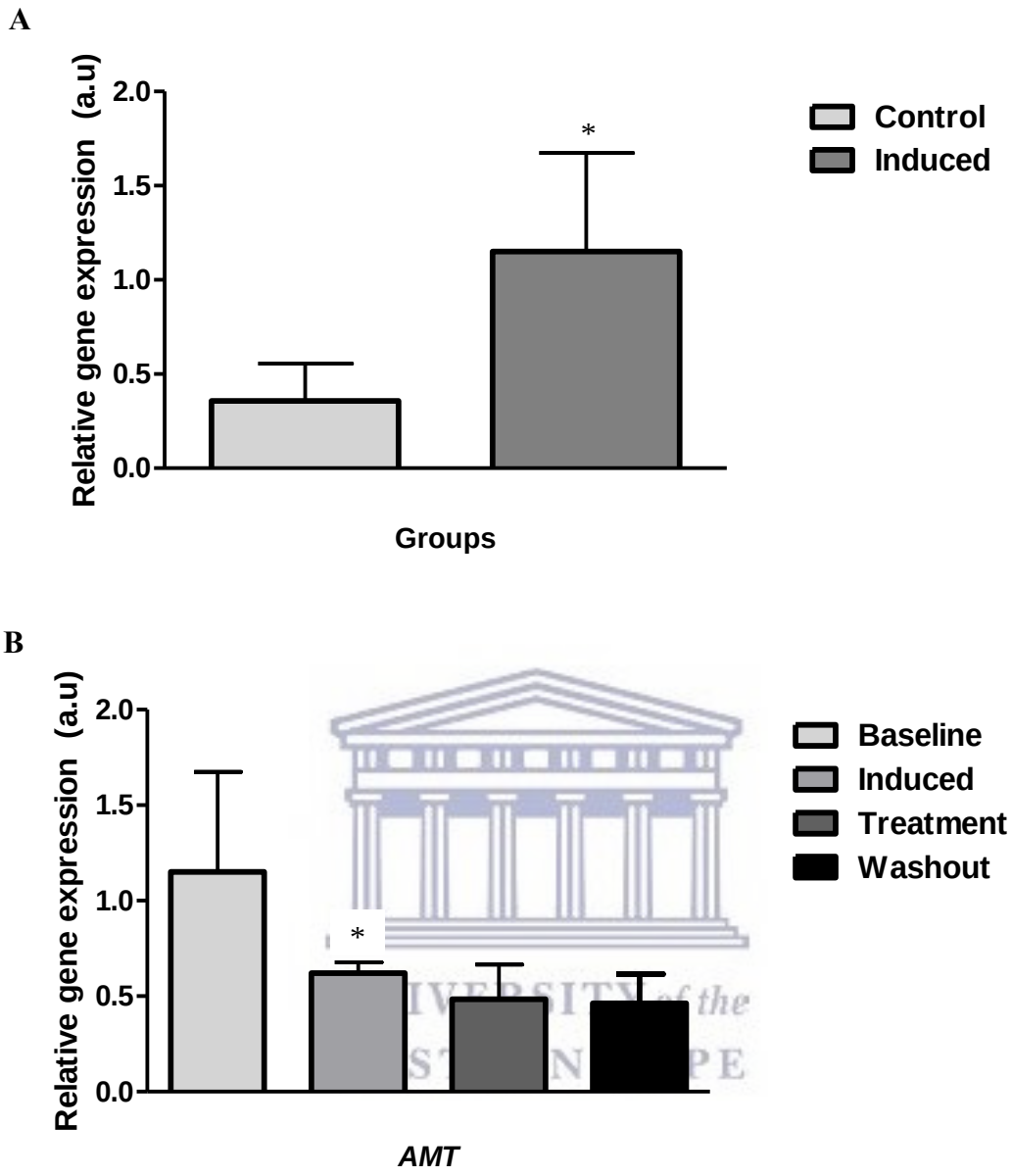


Figure 4.9: *AMT* mRNA gene expression. A) The expression of control compared to the induced group. The control group received a maintenance diet throughout the study while **t**he induced group received 50mg/kg/day of valproate and treated with sodium benzoate (250mg/kg/day) and dextromethorphan (5mg/kg/day) together with the spontaneous group. **B**) Expression of *AMT* at baseline, treatment followed by washout period of four weeks. The data was expressed as mean ± SD and mRNA expression in a.u. (arbitrary units). * represent significant difference ($P<0.05$)

4.4 DISCUSSION

In the current study, sequence changes were identified in *UGT1A6* and *UGT1A9* (Table 4.1) in the coding regions of exon 1, common exon 2 and 5. Additionally, four heterozygous sequence changes in *UGT1A6* (S7A, V15I, D51A, and T181A) and 12 in *UGT1A9* (M59I, D85V, K132M, Y189H, I193V, R208W, I211V, M212T, L219F, R222Y, A227I and P238S) were found in all the selected vervet monkeys including the controls. Since the vervet monkeys are captive-bred, it was challenging to determine whether the identified sequence changes were linked to a particular SNP or if they were mutations. Consequently, DNA samples from wild caught monkeys were included to verify the nature of these sequence variants. The sequencing findings showed that the changes were unique to the vervet model and were then considered as SNPs.

Common genetic alterations or polymorphisms, small insertions, deletions or SNPs within the genes encoding for the UGT can significantly affect the drug response of individuals (Ma and Lu, 2011). Mutations in exon one and promoter region affect the unique UGT isoforms and don't have direct impact on clinical conditions, while mutations located in the common exon 2-5 might simultaneously reduce the activity or expression of all UGTs (de Wildt et al., 1999, Mimura et al., 2011, Court et al., 2001). Gene expression of *UGT1A9* was compared between individual monkeys M104 and M113 for the following reasons: firstly, the presence of sequence variant in M104 and the lack thereof in M113, secondly, the positive drug response observed in M113 during valproate induction which was not observed in M104. At baseline, *UGT1A9* expression in M113 was higher than M104 and this was attributed to the identified sequence variants. Surprisingly, though M104 did not respond positively to valproate induction, an upward trend was observed during the induction period for gene expression (Figure 4.7), therefore, at a dose of 50mg/kg valproate

slightly altered the level expression of *UGT1A9* and findings varies per individuals. This partially explains why the findings in chapter three were not significant in elevating hyperglycinemia. According to Sun et al. (2017), individuals with heterozygous mutations in *UGT1A6* and *UGT1A9* tend to have lower valproate plasma concentration compared to homozygous carriers and this results in lower glucuronidation activity (Guo et al., 2012, Sun et al., 2017). A dose higher than 50 mg/kg was therefore required to induce hyperglycinemia in plasma for all the individuals with heterozygous sequence variants. Therefore, the identified sequence variants in exon one, three and five might have altered the function of *UGT1A6* and *UGT1A9* by interfering with valproate metabolism, hence induction was inconclusive in chapter three.

Among the identified sequence changes in *UGT1A6*, the S7A and T181A were corresponding to the polymorphisms that are reported in literature (Jain et al., 2015, Krishnaswamy et al., 2005, Munisamy et al., 2013). These polymorphisms are responsible for high toxicity in epileptic patients on valproate monotherapy treatment (Munisamy et al., 2013, Krishnaswamy et al., 2005). The human carriers with S7A, T181A and T184 sequence variants tend to have lower valproate serum levels compared to the wild type (Guo et al., 2012). On the contrary, the S7A, T181A and T184S polymorphism have been reported not to have any significant effect in patients treated with valproate (Chu et al., 2012, Wang et al., 2010, Jain et al., 2015). In this study, the effect of these polymorphisms on valproate concentration was not determined due to the lack of technical resources, expertise and financial constraints. However, it is possible that these sequence variants impaired the activity of the gene; hence valproate did not induce hyperglycinemia in all the selected monkeys because it may have been poorly metabolised. A slight up-regulation of *UGT1A9* expression was determined after valproate induction, however, conflicting findings were obtained

amongst the monkeys. Therefore, their genetic make-up played a role on how the individual monkeys responded to the induction.

Furthermore, the expression of *GLDC* and *AMT* was slightly reduced during the induction phase, this means that higher dose of the compound might show conclusive findings especially if it administered for a longer period. However, similar individual variations were observed for *GLDC* and *AMT* expression which further confirmed that valproate induction was responsible for inter-individual variations for both glycine and gene expression analysis. Furthermore, these variations are common in valproate treated individuals and this is mostly attributed to sequence variants in both *UGT1A6* and *UGT1A9* (Kuypers et al., 2005, Girard et al., 2004). Since this is the first time reporting sequence variants in nonhuman primates, the impact of these reported sequence changes may be similar to the ones observed in humans, thus affecting valproate metabolism and the functions of UGT.

4.5 CONCLUSION

Several novel variants were identified in *UGT1A6* and *UGT1A9*, although some variants were present in all the selected vervet monkeys. Gene expression of *UGT1A6*, *UGT1A9*, *GLDC* and *AMT* indicated inter-individual variations which correlated with glycine analysis, however, the expression findings were not significant throughout the study. It is therefore possible that the impact of the combined sequence variants in *UGT1A6* and *UGT1A9* genes may have been responsible for the inter-individual variations in glycine analysis and gene expression. Nevertheless, the inconclusive findings for valproate induction can be attributed to the heterozygous sequence variants in *UGT1A6* and *UGT1A9*.

CHAPTER FIVE:

Identification of sequence variants in GlyT1 and NKH genes in correlation with hyperglycinemia in a colony of captive-bred vervet monkey

5.1 INTRODUCTION

The GCS and NKH genes in correlation with hyperglycinemia has been well described in humans but the information for the GlyT1 gene is lacking. In the vervet monkey, mutations in the GCS components of vervet monkeys were also correlated with hyperglycinemia (Chauke et al., 2016). From earlier discussions we can hypothesise on the possibility that hyperglycinemia in this species may have been caused by the defective GCS component (*AMT*, *GLDC* and *GCSH*) or the gene encoding for GlyT1 (*SLC6A9*). At this point it is not clear whether cataract formation is the underlying factor for hyperglycinemia, though a correlation has previously been suggested (Chauke et al., 2016). Both NKH (*GLDC*, *AMT* and *GCSH*) and cataract (*GCNT2*) genes have been previously sequenced and mutations have been reported in these genes except for *GCSH* (Table 5.1), which is known to account for less than 1% of NKH cases (Kure et al., 2001). Therefore, mutations in *GLDC*, *AMT* and *GCNT2* genes were used as a reference for gene expression analysis since the same cataract animals were utilised for this study. The role of sequence variants in gene expression of GCS, GlyT1 and cataract genes will be determined, and the primary cause of both cataract formation and hyperglycinemia will be defined in these affected vervet monkeys.

Besides being localised in the glia, GlyT1 is expressed in the amacrine and ganglionic cells of the retina (Zafra et al., 1995, Vaney et al., 1998, Pow and Hendrickson, 1999, Pow, 1998, Avila et al.,

2013). The amacrine cells lose their glycine content when exposed to sarcosine, which is a competitive inhibitor for GlyT1. Glycine that is released by these cells competes for reuptake, thus leading to low levels of glycine in the neurons (Pow, 1998). Moreover, GlyT1 in caudal regions is responsible for the fast uptake of glycine and the terminating glycinergic inhibition, whereas GlyT1 in forebrain regions contributes to NMDAR neurotransmission. Signalling through NMDA receptors is higher in mice with reduced GlyT1 function, as a result, elevated glycine levels lead to functional changes in glutamatergic synapses as they exhibit enhanced NMDA transmission (Gabernet et al., 2004, Tsai et al., 2004).

Furthermore, high glycine concentration supplied through blood is found in the vitreous humor between the lens of an eye and the retina (Wright and Seggie, 1992). Since the lens of an eye contains high glycine content, they also possess SHMT activity and this influences the availability of glycine to retinal glycinergic neurons between the lens and vitreous humor especially in the absence of specific permeability barriers (Pow, 1998). Under normal circumstances, glycine in that vicinity is taken up by retinal neurons which are dependent on the glycine transporters for their glycine content (Pow, 1998). Thus, GlyT1 has two important roles in the retina which involves the normal cycling of glycine to support glycinergic inhibitory neurotransmission and to keep external glycine sufficiently low to avoid saturation of NMDA coagonist site (Stevens et al., 2010, Smith et al., 1992).

Thus far, defective GlyT1 has been reported in zebra fish (Cui et al., 2005, Hirata et al., 2010) and a mice models (Harvey et al., 2008, Gabernet et al., 2004, Gomeza et al., 2003, Tsai et al., 2004). Therefore, this study will be the first to report screening of GlyT1 gene in hyperglycinemic NHPs.

The glycine transporter belongs to the solute carrier (SLC6) family of sodium and chloride-dependent neurotransmitter and play a complementary role at glycinergic synapses (Eulenburg et al., 2005). In general, SLC6 regulates the transport of inorganic ions, nucleotides, amino acids, neurotransmitters and drug molecules across biological membranes (Hediger et al., 2004). The human *SLC6A9* encodes for five GlyT1 variants (a, b, c, d and e) (Harvey and Yee, 2013) and the loss of GlyT1 activity could underlie hyperglycinemia (Gomeza et al., 2003, Harvey and Yee, 2013).

In this study, the hyperglycinemic monkeys presented cataract formation (spontaneous), which is known to affect the normal function of the lens by interfering with the sharp focus of light in the retina (Graw et al., 2001). The onsets of the cataracts were detected microscopically at an age between three months to one year (de Villiers et al., 2001). Currently, there are no studies that directly link hyperglycinemia to cataract formation, however, an association has been suggested in vervet monkeys (Chauke et al., 2016). It has been reported that elevated serum glycine levels contribute to visual disturbance thus resulting in glycine's role as an inhibitory neurotransmitter in retina (Basnal et al., 2002, Karci and Erkin, 2003, Pérez-León et al., 2004, Barletta et al., 1994, Creel et al., 1987). In humans, hyperglycinemia has been reported to influence temporary vision loss (Tanaka et al., 1993) with the indication of high glycine levels on the retina (Basnal et al., 2002). On the contrary, administration of glycine in diabetic rats (Bahmani et al., 2012) and eye lenses of mammals (Rathore and Gupta, 2010) have shown to delay the onset and progression of vision impairment such as cataract. In a colony of vervet monkeys, it is obvious that glycine was not beneficial to the affected individuals as they are presented with progressing cataract phenotype.

Therefore, the role of glycine and its transporter was investigated in these cataract monkeys with spontaneous hyperglycinemia. Although GlyT1 mutations have been reported in zebra fish (Mongeon et al., 2008), there is no evidence of GlyTI genetic studies investigating its relation to hyperglycinemia in humans. Therefore, the findings from the nonhuman primate study are novel and contribute in the translation of future discoveries in human studies.

The purpose of this chapter was to confirm the involvement of GCS and GlyT1 on the development of hyperglycinemia in vervet monkeys with cataract formation. Gene expression was carried out for *GLDC*, *AMT* and *SLC6A9*. The latter gene was selected given that it regulates the activity of GlyT1, which has been associated with NKH in a knockout mouse model (Gomeza et al., 2003, Tsai et al., 2004). Whereas previous enquiries in these cataract vervet monkeys merely focused on genotyping (Chauke et al., 2016), the emphasis of this investigation broadens on the comparison of gene expression between GCS (*AMT* and *GLDC*) and cataract (*GCNT2*) genes. Additionally, genotyping and gene expression levels were correlated with sodium benzoate and dextromethorphan treatment. In chapter three, the glycine levels of the spontaneous group were normalised by the combination of sodium benzoate and dextromethorphan treatment. Therefore, the impact of the treatment with regards to expression of the selected genes was also determined. Gene expression assays developed for humans and rhesus macaque were employed to determine the effect of sequence changes in *GLDC*, *AMT* and *SLC6A9*.

5.2 MATERIALS AND METHODS

The monkeys were assigned into three groups, as described in Chapter two: group one (control), group two (induced) and group three (spontaneous: cataract & hyperglycinemia) consisting of four animals per group. This chapter focuses on the genotyping and the expression of the hyperglycinemic/cataract (spontaneous group) monkeys before and after the animal intervention (sodium benzoate and dextromethorphan treatment). The primers for twelve exons of *SLC6A9* (accession no: XM_002801515.1) were designed, synthesised (Appendix B, Table B1) and screened using PCR. The purified PCR products were sequenced and mutations analysed using CLC- DNA workbench, ExPasy translate tool and ClustalW. After careful selection of the candidate genes (*GLDC*, *AMT*, *SLC6A9*, and *GCNT2*) genotyping and gene expression protocols were standardised as described in Chapter two. The primer assays for all the genes were purchased, optimised and mRNA expression profiles were determined using qRT- PCR for both spontaneous group and control group. Two housekeeping genes (*PGK1* and *GAPDH*) were used to normalise mRNA levels of the prioritised genes and the control group was used as a calibrator.

5.3 RESULTS

5.3.1 Analysis of *SLC6A9* sequence variants

Sequence analysis of the *SLC6A9* coding region revealed only one transition silent mutation (C1419>T) in exon 11 (649bp) (Figure 5.1 and 5.2). This heterozygous nucleotide change is located in codon 473 (A473A) and is in a conserved region when aligned with human and rhesus reference sequence.

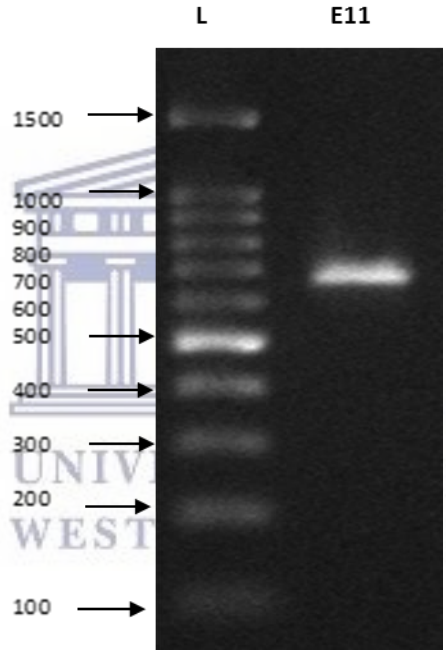


Figure 5.1: *SLC6A9* genetic analysis of selected captive-bred vervet monkeys in the spontaneous group. A 2% agarose gel electrophoresis showing exon 11 of *SLC6A9* (649bp) gene. M = 100-1500bp DNA ladder.

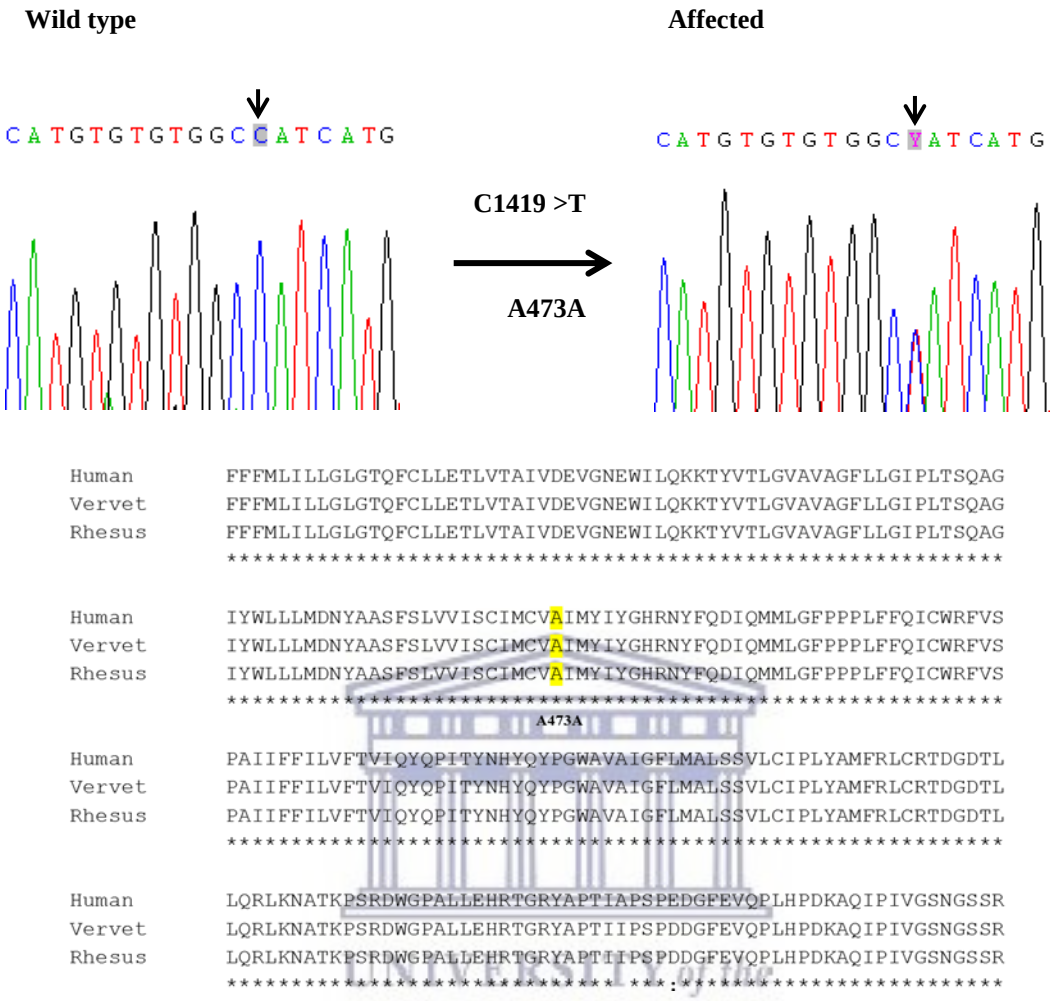


Figure 5.2: *SLC6A9* mRNA sequence alignment between human, vervet and rhesus species using ClustalW. A) Sequence chromatogram displaying heterozygous transition silent mutation (C1419>T) in exon 11 of *SLC6A9*. The change at codon 473 (A473A) was present in the spontaneous group but not in the control group. The sequence highlighted yellow represent the variant. Below the protein sequences is a key denoting conserved sequence (*), conservative mutations (:), semi-conservative mutations (.), and non-conservative mutations ( ).

5.3.2 Analysis of *SLC6A9* gene expression

A significant difference (P-value = 0.02) was observed during intervention phase. At baseline, hyperglycinemia reduced the expression of *SLC6A9* gene in the spontaneous group compared to the control group (Figure 5.3A). However, the expression was significantly elevated by sodium

benzoate (250mg/kg) and dextromethorphan (5mg/kg) treatment, followed by a significant decline during the washout period (Figure 5.3B).

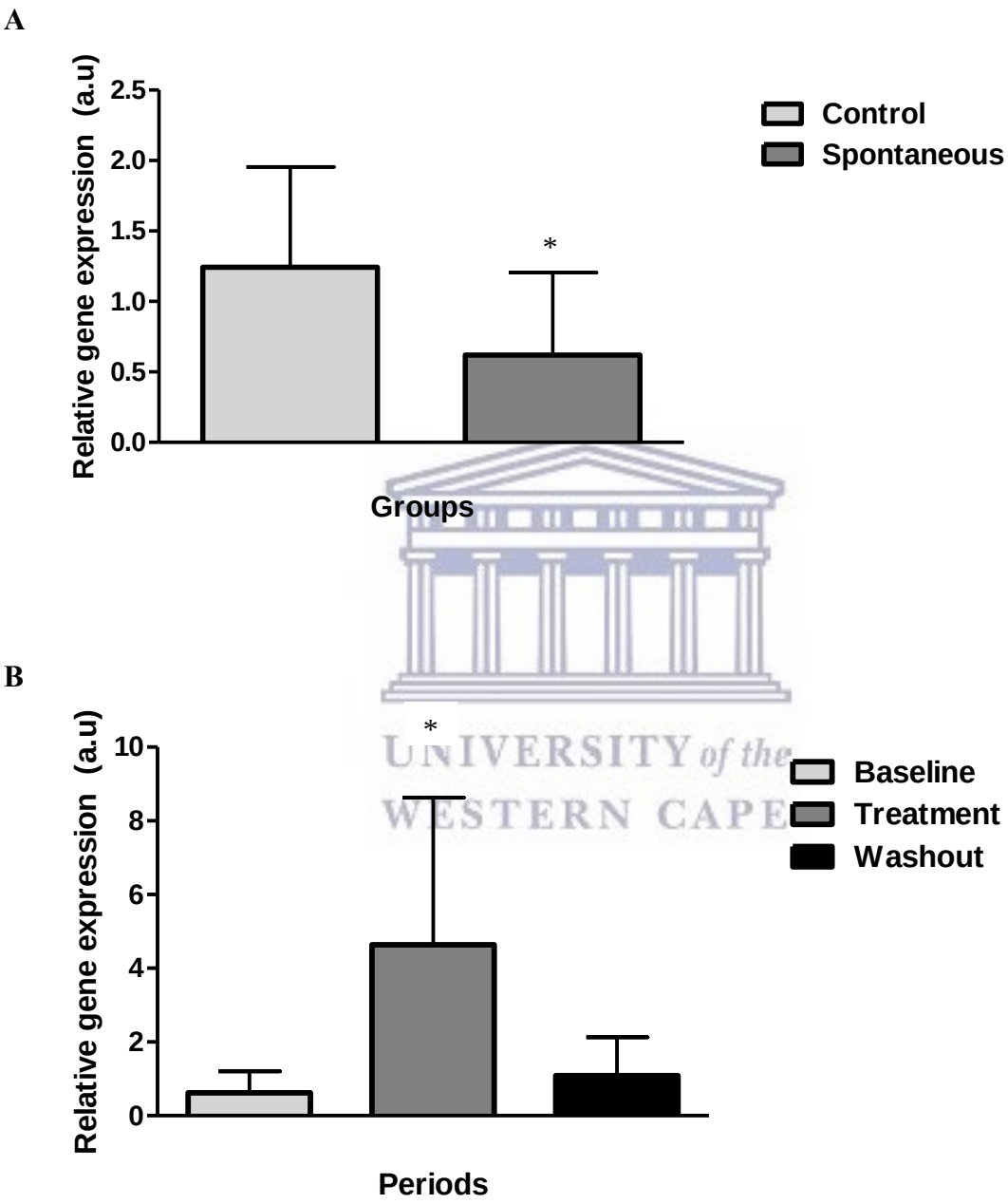


Figure 5.3: *SLC6A9* mRNA gene expression. A) The expression of control compared to spontaneous group. The control group received a maintenance diet throughout the study while the spontaneous group was treated with sodium benzoate (250mg/kg/day) and dextromethorphan (5mg/kg/day) for four weeks. **B)** Expression of *SLC6A9* at baseline, treatment followed by washout period of four weeks. The data was expressed as mean ± SD and mRNA expression in a.u. (arbitrary units). The * represent significant difference ($p < 0.05$).

5.3.3 Analysis of sequence variants and gene expression for *GLDC* and *AMT*

Sequence variants in *AMT* (G46G) and *GLDC* (S44R, L101S, G135D, T269T, S617S and T642M) were identified in the spontaneous individuals with the only exception of one silent mutation (S617S) in the controls (Chauke et al., 2016) (Table 5.1). A significant difference between the control and the spontaneous group was observed at baseline (p-value < 0.05) for both *GLDC* (Figure 5.4A) and *AMT* (Figure 5.5A). The expression of *GLDC* (Figure 5.4B) and *AMT* (Figure 5.5B) decreased during the treatment (25.4%, 11.3%) and washout period (35%, 24%) respectively, however a statistical difference was not observed for *AMT*.

Table 5.1: Previously identified sequence variants in *GCNT2*, *GLDC* and *AMT*

Gene	Exon	Nucleotide change	Type of change	Amino acid change	Type of mutation	No. of mutation carriers	
						Cataract (N=12)	Control (N=12)
***GLDC**	1	C132A	Transversion	S44R	Missense	2	0
	2	T302C	Transition	L101S	Missense	2	0
	3	G404A	Transition	G135D	Missense	2	0
	7	G807A	Transition	T269T	Silent	1	0
	18	C1851T	Transition	S617S	Silent	12	12
	18	C1925T	Transition	T642M	Missense	4	0
***AMT**	2	C138T	Transition	G46G	Silent	8	0
GCNT2	1A	C772T	Transition	M258V	Missense	5	2
	1B	G46A	Transition	V16I	Missense	4	1
	1C	G75A	Transition	S24N	Missense	1	0

*****Adapted from (Chauke et al., 2016)

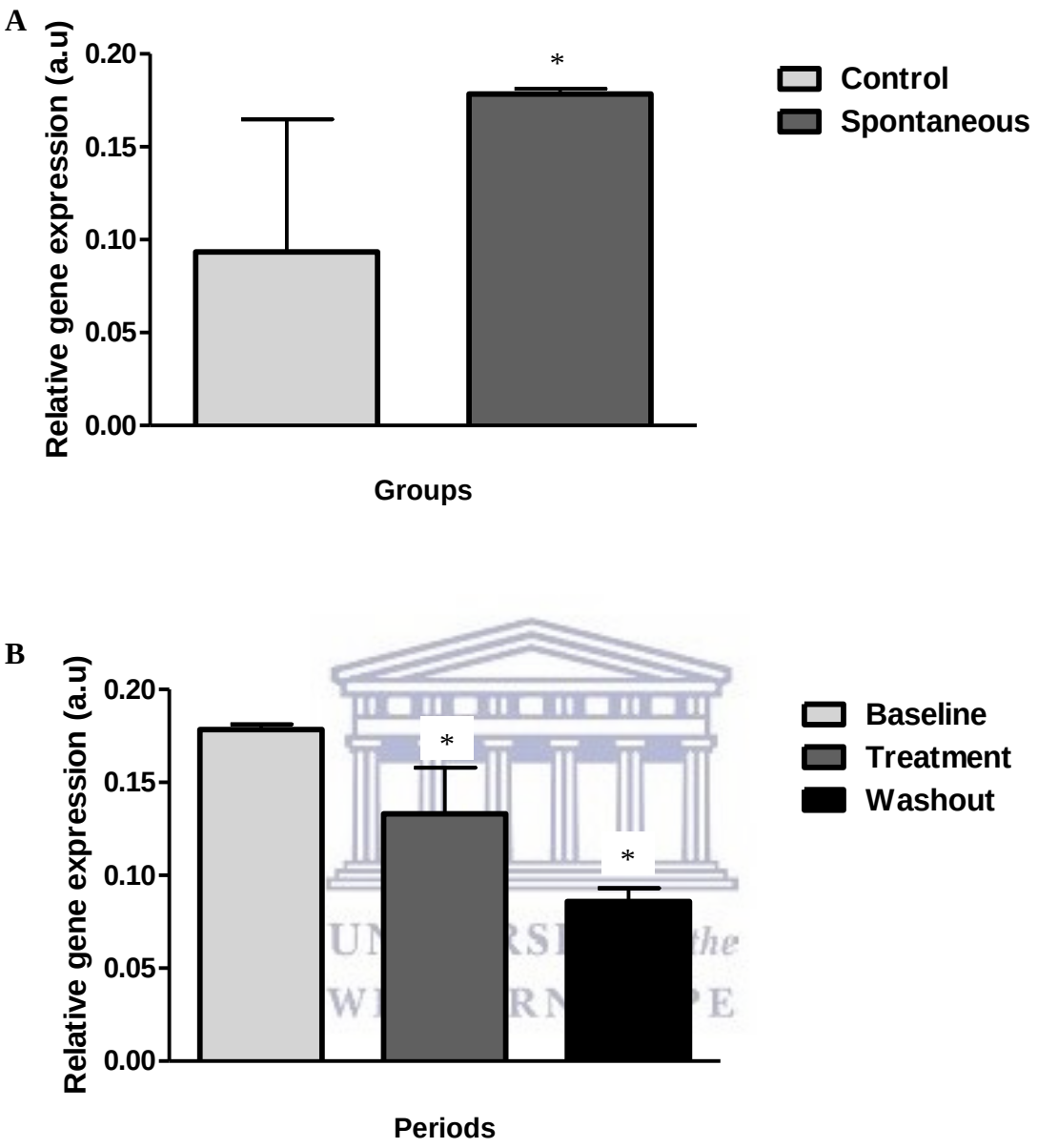


Figure 5.4: *GLDC* mRNA gene expression. A) The expression of control compared to spontaneous group. The control group received a maintenance diet throughout the study while the spontaneous group was treated with sodium benzoate (250mg/kg/day) and dextromethorphan (5mg/kg/day) for four weeks. **B)** Expression of *GLDC* at baseline, treatment followed by washout period of four weeks. The data was expressed as mean ± SD and mRNA expression in a.u. (arbitrary units). * represent significant difference ($p < 0.05$).

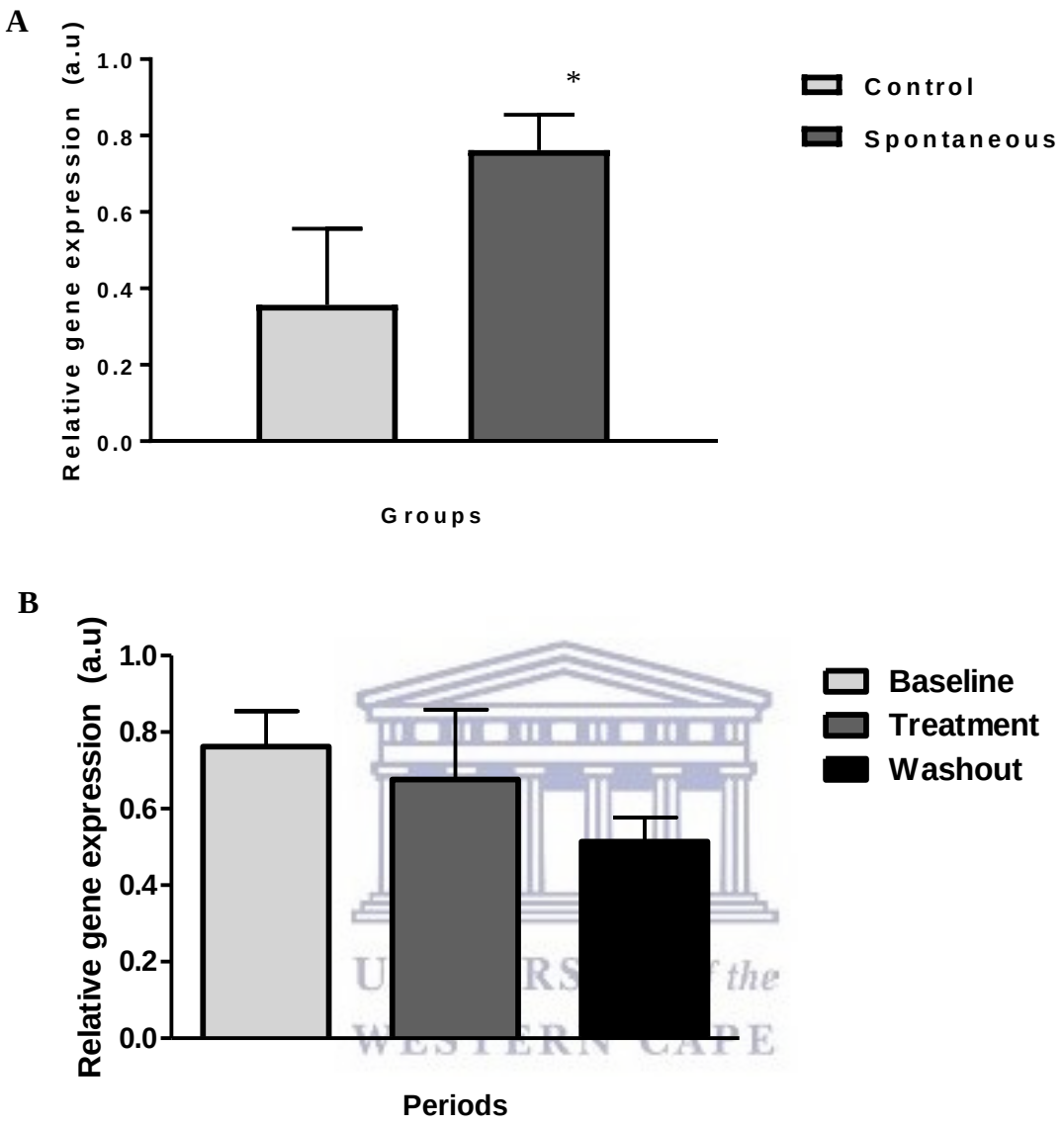


Figure 5.5: *AMT* mRNA gene expression. A) The expression of control compared to spontaneous group**.** The control group received a maintenance diet throughout the study while the spontaneous group was treated with sodium benzoate (250mg/kg/day) and dextromethorphan (5mg/kg/day) for four weeks. **B)** Expression of *AMT* at baseline, treatment followed by washout period of four weeks. The data was expressed as mean ± SD and mRNA expression in a.u. (arbitrary units). * represent significant difference ($p < 0.05$).

5.3.4 Comparison of expression between cataract and NKH genes

Congenital cataract genes were previously sequenced in the spontaneous group (unpublished data) (Table 5.1). Gene expression findings were compared between *GLDC*, *AMT*, *SLC6A9* and *GCNT2* to confirm if the pattern of expression in the spontaneous group differs from the control. At baseline, differences in gene expression between the control and spontaneous group for *AMT* and *SLC6A9* were observed (Figure 5.6). The *AMT* gene was up-regulated in the spontaneous individuals while *SLC6A9* was down regulated. Similar expression was observed between *GLDC* and *GCNT2*, where the former was highly expressed in the spontaneous group (Figure 5.7).

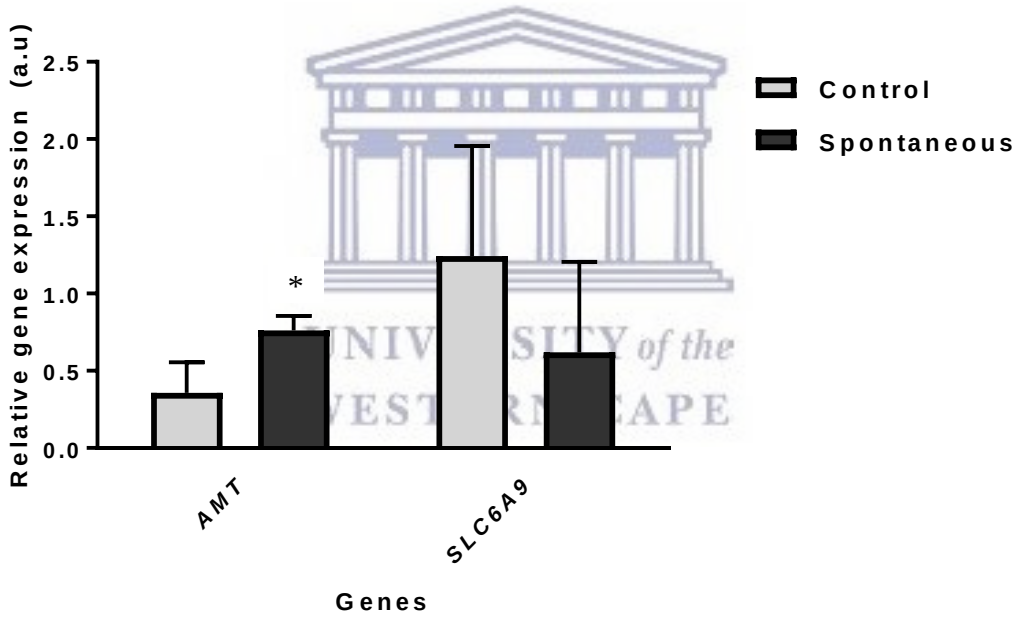


Figure 5.6: Baseline *AMT* and *SLC6A9* mRNA gene expression in spontaneous group compared to control group. The spontaneous group had cataract and hyperglycinemia, while the control group were cataract free with normal glycine levels. The data was expressed as mean ± SD and mRNA expression in a.u. (arbitrary units), those without error bars the SD was borderline to zero. * represent significant difference ($p < 0.05$).

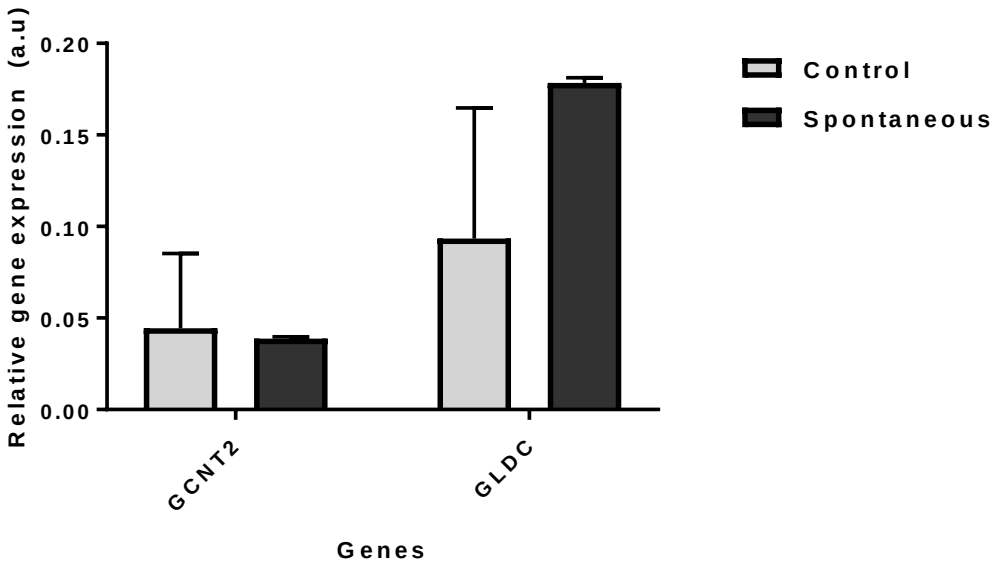


Figure 5.7: Baseline *GCNT2* and *GLDC* mRNA gene expression in spontaneous group compared to control group. The spontaneous group had cataract and hyperglycinemia, while the control group were cataract free with normal glycine levels. The data was expressed as mean ± SD and mRNA expression in a.u. (arbitrary units), those without error bars the SD was borderline to zero. * represent significant difference ($p < 0.05$).

5.4 DISCUSSION

The discovery of hyperglycinemia in our colony of captive bred vervet monkeys offered an opportunity to investigate this disease which was not previously reported in nonhuman primates. Although glycine transporters have not been studied in NKH patients, the findings from knockout mice model suggested that there is a possibility that mutations in GlyT1 gene (*SLC6A9*) might be the cause of NKH. However, the main features of NKH such as elevated CSF and plasma glycine have not been studied in rodents. In the present study, glycine levels in CSF and plasma were determined. The GCS and GlyT1 genes were included to screen for mutations and correlation with gene expression. These prioritized candidate genes, their sequence variants and expression levels were examined for possible association with the cataract genes. Therefore, findings from this study

might assist in establishing a patient profile for human studies since mutations and expression studies has not been reported for GlyT1 in association with NKH.

As it was mentioned, GlyT1 is expressed in the retina and regions of CNS where glycine act as a neurotransmitter (Zafra et al., 1995). It is hypothesised that GlyT1 gene (*SLC6A9*) might be responsible for another type of NKH, which has not been characterized (Gomeza et al., 2003). Furthermore, it is reported that 50% of NKH patients lack mutations in GCS and the complex is found to be normal is certain individuals with elevated glycine levels (Applegarth and Toone, 2001). There are also reports on NKH patients with normal GCS activity but defective glycine transport in post-mortem tissues (Applegarth and Toone, 2006). In such cases, the loss of glial GlyT1 uptake would be expected to cause elevated CSF glycine, while loss of GlyT1 from the peripheral sites could also lead to high serum glycine (Jursky and Nelson, 1996). Since both CSF and plasma glycine were elevated in spontaneous group, this means GlyT1 was not functioning in both glial and peripheral sites.

In this study, sequencing of GlyT1 gene (*SLC6A9*) indicated one novel silent mutation (A473A) which was present only in the spontaneous group. This variant is located in highly conserved tenth transmembrane region when aligned with previously reported studies (Cui et al., 2005, Harvey et al., 2008). Since silent mutations in coding exons are known to modify gene expression (Zhang et al., 2012), a possibility exists that this sequence variant could have contributed to the reduced expression in the spontaneous group. However, it could also be possible that certain variants such as large scale deletions and rearrangements were overlooked due to the shortfalls of sequencing which only detects small scale variants (Herman et al., 2012, Hogervorst et al., 2003).

Furthermore, Cui et al., 2005 identified a missense mutation (G81D) in zebrafish GlyT1 (*slc6a9*) which was located in a highly conserved transmembrane region. The G81D severely compromised the activity of GlyT1 thus resulting in elevated glycine concentration in CNS. It is therefore, possible that the underlying mutations that contributed to high glycine levels in the vervet monkeys might have been overlooked in this study due to the sequencing method that was selected. It is therefore recommended to use more advanced techniques such as multiplex ligation-dependent probe amplification (MLPA) (Kanno et al., 2007). The MLPA can detect large deletions which are often missed when using Sanger sequencing. It is possible that hyperglycinemia in the spontaneous group was due to the defective clearance of glycine by GlyT1. Evidently, high CSF glycine levels occur due to a mutation in GlyT1 that regulates the uptake of glycine from glial cells (Gomeza et al., 2003). Thus leading to chronic inhibition at glycinergic synapse and increased excitation at NMDA synapses in GlyT1 (Cui et al., 2005). The potentiation of inhibitory glycinergic transmission which is likely responsible for postnatal death of GlyT1 knockout mice (Gomeza et al., 2003) was observed in mutated (G81D) zebrafish. These observations suggested that GlyT1 plays an important role in terminating glycinergic transmission by removing glycine from synaptic cleft (Gomeza et al., 2003, Cai et al., 2006).

At this stage, it can only be speculated that defective GlyT1 could have been the cause of death in the vervet neonates that died immediately after birth, however, their glycine status was not evaluated although they were siblings of the hyperglycinemic affected individuals. Similarly, a defective GlyT1 is known to be responsible for death after birth (Gomeza et al., 2003; Tsai et al., 2004), however, in some cases the knockout mice can survive without showing symptoms of NKH (Eulenburg et al., 2010). Therefore, it is possible that the mutated *SLC6A9* might have contributed

to the death of those vervet neonates that were found dead. Furthermore, a reduction in the transporter uptake activity correlates with the reduced protein expression (Gomeza et al., 2003, Cai et al., 2006, Eulenburg et al., 2010). The phenomenon of reduced expression was observed in the spontaneous group where *SLC6A9* expression was lower than the control (Figure 5.3A). Additionally, sodium benzoate and dextromethorphan was seen to normalised glycine levels in the spontaneous group which significantly up-regulated the expression of *SLC6A9* (Figure 5.3B).

Though the treatment elevated *SLC6A9* expression, a different pattern was observed for *GLDC* and *AMT* where the treatment reduced the expression of both genes in the spontaneous group. Even though, the expression of *SLC6A9* at baseline was similar to the cataract gene (*GCNT2*), which was also suppressed in the spontaneous group, the findings between *SLC6A9* and *GCNT2* suggested that GlyT1 might also be associated with cataract formation in the colony. This is also supported by the expression of GlyT1 in the eye lens and retina (Zafra et al., 1995), however extensive investigation using a larger spontaneous group size is critical.

It has been established that hyperglycinemia and the combined treatment with sodium benzoate and dextromethorphan demonstrated contradicting effect on GCS and GlyT1 even though both the complex and the glycine transporter function to regulate extracellular glycine concentration. Therefore, both GCS and GlyT1 might have contributed to the elevated glycine levels in the spontaneous group despite the differences in expression. Significantly, the findings of *SLC6A9* in nonhuman primate model will contribute to the existing literature on the topic of GlyT1 mutations and gene expression. Thus opening a research forum that will link the glycine transporter to NKH not just in rodents and zebrafish but in humans as well.

5.5 CONCLUSION

In this study, hyperglycinemia in a colony of vervet monkeys was associated with both defective GCS components (*GLDC* and *AMT*) and GlyT1 (*SLC6A9*) genes which have been sequenced to identify the disease-causative sequence variants. The findings indicated that even though the silent variant might have a low impact on *SLC6A9*, it does not rule out the fact that defective GlyT1 might have contributed to the development of hyperglycinemia in vervet monkeys. Underlying disease-causing variants might have been overlooked and therefore, recommended that more advanced techniques such as MLPA must be used for future studies. Nevertheless, it can be concluded that high glycine levels in the spontaneous group either elevates or suppress expression levels of *GLDC*, *AMT*, *SLC6A9* and *GCNT2*. Additionally, the findings from this study supports the suggested correlation between cataract formation and hyperglycinemia in this colony of captive-bred vervet monkeys. However, due to a small sample size that was used for both intervention and gene expression experiments, a larger sample size will be required for future genotyping and gene expression studies.

CHAPTER SIX:

General discussion and conclusions

6.1 DISCUSSION

Hyperglycinemia was observed for the first time in the colony of captive-bred vervet monkeys. The discovery of high glycine levels in CSF and plasma of cataract-affected individuals offered an opportunity to investigate the underlying cause of both cataract and hyperglycinemia. The presence of hyperglycinemia and cataract formation in these monkeys is unique since both disorders have never been reported concurrently. In this case, the affected monkeys lacked the clinical signs of NKH such as lethargy, hypotonia, seizures and apnea which are common in human neonates (Boneh et al., 2005). However, jerky movements have been observed in some of the surviving siblings which occurred until adulthood and neonatal deaths from cataract related monkeys have been reported within the colony. The cause of death was from head trauma after speculated seizure attacks and hypotonia which resulted in neonates failing to clutch on their mothers.

Similarly, death after birth has been reported in a knockout mice model with a defective GlyT1 protein (Gomeza et al., 2003; Tsai et al., 2004). In some cases, the GlyT1 knockout mice survived adulthood without showing symptoms of NKH (Eulenburg et al., 2010). In this study, 8% of the hyperglycinemic monkeys survived until adulthood and it can only be speculated that defective uptake of glycine might have contributed to those neonates that were found dead. Therefore, the presence of hyperglycinemia in this colony makes the vervet monkey an ideal animal model to further evaluate the involvement of GlyT1 in NKH.

The purpose of the study was to determine and normalise glycine levels in hyperglycinemic cataract monkeys, which was achieved using two approaches. The first approach was animal intervention where valproate (50mg/kg) was used to induce hyperglycinemia. Thereafter, a combination of sodium benzoate (250mg/kg) and dextromethorphan (5mg/kg) was used to normalise glycine levels in CSF and plasma. The second approach focused on the molecular aspects of NKH using genotyping and gene expression of UGTs, NKH, GlyT1 and cataract genes.

6.1.1 Animal intervention approach

Phase one of the intervention study was achieved by elevating glycine level through valproate induction which was administered orally and consumed voluntarily. This anticonvulsant has been used for years to treat epilepsy (Avery and Bumpus, 2014, Ahmed and Siddiqi, 2006) and has been reported to elevate glycine levels in humans (Morrison et al., 2006) and vervet monkeys (Viljoen et al., 2012). The induction phase was included to determine the effectiveness of NKH treatment when compared to the spontaneous model. Furthermore, the combined treatment of sodium benzoate and dextromethorphan was also administered orally to decrease glycine levels in plasma and CSF of both induced and spontaneous monkeys.

The effect of these three compounds in biochemical, haematology parameters and glycine levels were monitored throughout the study. In phase one, valproate caused changes at a dose of 50 mg/kg in alkaline phosphatase, phosphate and platelet count. While in phase two, the treatment reversed the valproate effect in those affected parameters and normalised glycine levels (<350 µmol/L) in both induced and spontaneous groups. However, the treatment was more effective in reducing

glycine levels in the spontaneous group compared to induced group. The induction and treatment outcomes in the induced group were not satisfactory, it is therefore, suggested that a higher dose of valproate (>50 mg/kg) and the treatment with sodium benzoate (>250 mg/kg) and dextromethorphan (>5 mg/kg) must be considered in order to induce and treat hyperglycinemia, respectively. Although there are conflicting findings about the effectiveness of NKH treatment in humans (Neuberger et al., 2000, Randak et al., 2000, Van Hove et al., 2005) with some claims of the treatment not being effective in reducing glycine levels especially in CSF (Lu et al., 1999, Madu and Oliver, 2013), findings from this study indicated that the combination treatment of sodium benzoate (250 mg/kg) and dextromethorphan (5 mg/kg) was effective in normalising glycine levels in the spontaneous group of captive-bred vervet monkeys.

6.1.2 Genotyping and gene expression approach

The same study groups were used for the molecular experiments which focused on genotyping and gene expression. Out of the 15 sequence variants identified in chapter four, two variants (S7A, T181A) in *UGT1A6* have been reported to be responsible for valproate toxicity in epileptic patients (Munisamy et al., 2013, Krishnaswamy et al., 2005). Individuals with S7A and T181A sequence variants tend to require a higher dose of valproate compared to wild type (Guo et al., 2012). Therefore, it is possible that both S7A and T181A had an impact on how the induced group responded to valproate induction thus affecting gene expression. However, there are conflicting outcomes about the impact of these variants in humans (Chu et al., 2012, Wang et al., 2010, Jain et al., 2015). In this study, biochemistry and haematology differences were observed for Mg, LDL, urea, MCV between the study groups, however, ALP, phosphate and platelet count were only observed in the induced group. The latter group did not respond to induction and this was due to

sequence variants that were identified in exon one, three and five. Consequently, gene expression of *UGT1A6* was inconclusive and this might explain why glycine was not elevated in chapter three. With reference to *GLDC* and *AMT* genes, valproate induction significantly reduced the expression of *AMT* compared to *GLDC* and remained low for both genes during treatment and washout period.

A previous investigation suggested that mutations in both *GLDC* and *AMT* genes were responsible for hyperglycinemia that was observed in the colony (Chauke et al., 2016). The treatment significantly downregulated the expression of *GLDC* compared to *AMT*. However, a different expression profile was observed for *SLC6A9,* which was elevated by the treatment and returned to baseline levels after washout. Since defective glycine transporters are known to contribute to glycine accumulation, the findings in *SLC6A9* suggest that GlyT1 activity was impaired in the spontaneous group which resulted in elevated glycine levels in plasma and CSF. It can be concluded that *GLDC*, *AMT* and *SLC6A9* may be responsible for hyperglycinemia in CSF and plasma of the cataract monkeys.

The following study objectives were therefore achieved:

- Elevated glycine levels in CSF and plasma were found in group three (spontaneous cataract/hyperglycinemic) compared to group one (control) and group two (induced).
- The pharmacodynamics of valproate, sodium benzoate and dextromethorphan were determined in the experimental groups. There were no signs of liver or kidney disease throughout the study although valproate elevated the levels of ALP, phosphate and platelet count. Additionally, sodium benzoate and dextromethorphan treatment reversed the effect

of valproate in biochemical and haematological parameters and it was more effective in normalising glycine levels in group three than in group two.

- The response from animal intervention was correlated with sequence variants identified in *UGT1A6*, *UGT1A9* and *SLC6A9*. It is possible that the combined effect of sequence variants in *UGT1A6* and *UGT1A9* had an impact on the overall activity of the two UGT isoforms hence the *UGT1A6* gene expression was inconclusive as it could only be determined in baseline samples but not in treated samples.
- The effect of valproate, sodium benzoate and dextromethorphan on a gene expression was minimal except for *SLC6A9* which was significantly up-regulated by sodium benzoate and dextromethorphan.

The limitations of this study included:

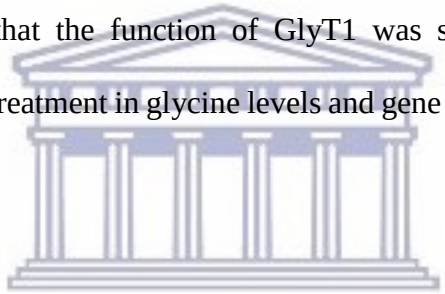
- Only a small group of animals were available due to ethical and financial constraints. Thus, only twelve monkeys were accessible for this study. Consequently, the intended in depth analysis on the effect of valproate, sodium benzoate and dextromethorphan in tissue samples could not be achieved due to financial constraints and ethical issues.
- During the study, the complete vervet genome was not available on the Ensembl database and consequently impacted on the primer design for the prioritized genes. Alternatively, rhesus sequences were adopted as a reference.

Therefore, the data presented in the current investigation on hyperglycinemia forms the basis of what could become a much larger comparative study. Additional surveys with increased sample size for genotyping are required to confirm sequence variants that were identified. Specific focus

should be targeted on future correlation analysis to validate the findings of this study. This study revealed that sodium benzoate and dextromethorphan were effective in treating hyperglycinemia. Finally, the relationship between GlyT1 and NKH was suggested in this colony with specific reference to cataract formation and hyperglycinemia in CSF and plasma.

6.2 CONCLUSION

The effect of the valproate induction and the combined treatment of sodium benzoate and dextromethorphan appeared to have minimal effect on gene expression of *AMT* and *GLDC* except for *SLC6A9*. Gene expression findings for *SLC6A9* was correlated with high glycine levels in CSF and plasma which suggested that the function of GlyT1 was suppressed. The findings also confirmed that the effect of the treatment in glycine levels and gene expression was only reversible in the spontaneous group.



This study confirmed for the first time that the genotyping and gene expression changes in *SLC6A9, AMT* and *GLDC* might have contributed to the development of hyperglycinemia in cataract vervet monkeys. Additionally, the findings can also be extrapolated to humans since GlyT1 involvement has not been reported.

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APPENDIX A: Animal intervention

1. BIOCHEMICAL AND HAEMATOLOGICAL RAW DATA

Table 1A: Biochemistry analysis during baseline, induction, treatment and washout

Parameter	Time point	Control	Induced	Spontaneous	P-value
AST	Baseline	79.50 ± 38.46	184.25 ± 195.91	52.00 ± 17.61	0.09
	Induction	N/A	125.33 ± 54.22	N/A	N/A
	Treatment	75.75 ± 25.81	68.75 ± 33.03	42.00 ± 11.34	0.87
	Washout	65.25 ± 14.89	109.00 ± 128.05	48.00 ± 22.73	0.46
ALT	Baseline	76.25 ± 10.53	158.75 ± 101.61	74.00 ± 12.68	0.28
	Induction	N/A	95.67 ± 72.86	N/A	N/A
	Treatment	84.50 ± 24.47	100.00 ± 66.82	92.50 ± 21.99	0.18
	Washout	89.00 ± 18.29	107.00 ± 43.35	82.50 ± 10.88	0.52
ALP	Baseline	194.50 ± 154.51	233.25 ± 78.33	134.00 ± 46.28	0.42
	Induction	N/A	357.33 ±142.63	N/A	N/A
	Treatment	214.25 ± 163.89	314.50 ± 101.36	181.00 ± 38.58	0.27
	Washout	205.00 ± 130.14	324.25 ± 76.77	184.50 ± 21.44	0.08
GGT	Baseline	56.00 ± 22.85	46.75 ± 11.21	52.50 ± 13.48	0.73
	Induction	N/A	65.00 ± 22.00	N/A	N/A
	Treatment	86.75 ± 45.51	78.00 ± 24.59	82.00 ± 19.41	0.92
	Washout	79.75 ± 37.43	65.00 ± 16.02	74.00 ± 20.90	0.73
Total protein	Baseline	59.50 ± 2.38	56.50 ± 2.08	57.25 ± 1.26	0.13
	Induction	N/A	62.00 ± 1.73	N/A	N/A
	Treatment	60.25 ± 1.71	61.00 ± 2.00	58.00 ± 2.45	0.15
	Washout	59.25 ± 1.89	57.00 ± 0.82	57.75 ± 3.86	0.47
Total bilirubin	Baseline	3.00 ± 0.82	3.00 ± 0.82	2.25 ± 0.50	0.29
	Induction	N/A	N/A	N/A	N/A
	Treatment	2.50 ± 0.58	2.50 ± 0.58	2.50 ± 0.58	0.40
	Washout	3.50 ± 0.58	3.25 ± 1.50	3.00 ± 0.82	0.79
Direct bilirubin	Baseline	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	ND
	Induction	N/A	N/A	N/A	N/A
	Treatment	1.00 ± 0.00	1.25 ± 0.50	1.00 ± 0.00	ND
	Washout	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	ND
Indirect bilirubin	Baseline	2.00 ± 0.82	2.00 ± 0.82	1.25 ± 0.50	0.29
	Induction	N/A	N/A	N/A	N/A
	Treatment	1.50 ± 0.58	1.25 ± 0.96	1.50 ± 0.58	0.85
	Washout	2.50 ± 0.58	2.25 ± 1.50	2.00 ± 0.82	0.79
Albumin	Baseline	31.25 ± 3.10	28.00 ± 3.92	31.75 ± 0.96	0.20
	Induction	N/A	36.33 ± 2.08	N/A	N/A
	Treatment	38.25 ± 1.26	38.50 ± 4.12	38.75 ± 2.06	0.96
	Washout	38.00 ± 0.82	37.00 ± 2.94	38.00 ± 1.83	0.73
Globulin	Baseline	28.25 ± 2.36	28.50 ± 5.20	25.50 ± 2.08	0.44
	Induction	N/A	25.67 ± 2.31	N/A	N/A
	Treatment	22.00 ± 1.83	22.50 ± 2.52	19.25 ± 3.95	0.28
	Washout	21.25 ± 1.89	20.00 ± 2.71	19.75 ± 4.99	0.80

Parameter	Time point	Control	Induced	Spontaneous	P-value
Cholesterol	Baseline	4.63 ± 0.50	4.65 ± 1.07	3.93 ± 0.39	0.31
	Induction	N/A	5.40 ± 0.53	N/A	N/A
	Treatment	5.10 ± 0.72	5.80 ± 0.82	4.28 ± 1.11	0.10
	Washout	4.95 ± 0.85	4.63 ± 0.51	4.20 ± 0.37	0.27
HDL-C	Baseline	2.01 ± 0.33	1.87 ± 0.29	1.93 ± 0.19	0.78
	Induction	N/A	2.03 ± 0.35	N/A	N/A
	Treatment	2.20 ± 0.42	2.28 ± 0.54	2.13 ± 0.55	0.91
	Washout	2.18 ± 0.30	1.90 ± 0.29	2.10 ± 0.22	0.37
LDL-C	Baseline	1.88 ± 0.54	2.05 ± 0.90	1.43 ± 0.24	0.37
	Induction	N/A	2.97 ± 0.32	N/A	N/A
	Treatment	2.50 ± 0.63	3.15 ± 0.66	1.78 ± 0.64	0.04
	Washout	2.60 ± 0.85	2.18 ± 0.51	1.80 ± 0.22	0.21
Urea	Baseline	3.43 ± 0.46	4.88 ± 0.87	3.48 ± 0.60	0.02
	Induction	N/A	7.70 ± 0.53	N/A	N/A
	Treatment	5.23 ± 1.09	7.85 ± 2.88	6.70 ± 1.93	0.25
	Washout	4.98 ± 0.51	8.95 ± 2.71	7.90 ± 2.26	0.05
Creatinine	Baseline	71.50 ± 12.56	64.00 ± 5.60	66.50 ± 11.70	0.60
	Induction	N/A	68.67 ± 6.43	N/A	N/A
	Treatment	59.75 ± 19.31	68.75 ± 9.18	64.50 ± 11.27	0.67
	Washout	67.75 ± 14.77	66.25 ± 15.22	72.75 ± 9.50	0.77
Triglycerides	Baseline	1.59 ± 0.66	1.45 ± 0.64	0.94 ± 0.14	0.25
	Induction	N/A	0.83 ± 0.21	N/A	N/A
	Treatment	0.74 ± 0.29	0.80 ± 0.36	0.46 ± 0.26	0.28
	Washout	0.53 ± 0.11	1.20 ± 0.90	0.66 ± 0.22	0.23

Parameter	Time point	Control	Induced	Spontaneous	P-value
Ca	Baseline	2.11 ± 0.19	2.09 ± 0.12	2.11 ± 0.06	0.96
	Induction	N/A	2.20 ± 0.07	N/A	N/A
	Treatment	2.14 ± 0.09	2.21 ± 0.11	2.16 ± 0.03	0.49
	Washout	2.11 ± 0.08	2.20 ± 0.07	2.17 ± 0.12	0.36
Na	Baseline	146.00 ± 2.16	145.00 ± 2.16	148.50 ± 0.58	0.06
	Induction	N/A	149.67 ± 0.58	N/A	N/A
	Treatment	148.25 ± 1.26	148.25 ± 1.71	145.75 ± 6.50	0.53
	Washout	145.25 ± 0.96	146.00 ± 3.16	149.75 ± 0.50	0.06
Mg	Baseline	0.61 ± 0.04	0.62 ± 0.03	0.59 ± 0.05	0.69
	Induction	N/A	0.81 ± 0.04	N/A	N/A
	Treatment	0.74 ± 0.04	0.82 ± 0.03	0.70 ± 0.03	0.001
	Washout	0.70 ± 0.04	0.75 ± 0.06	0.69 ± 0.04	0.18
Cl^-	Baseline	106.00 ± 2.16	106.00 ± 2.31	109.00 ± 0.82	0.08
	Induction	N/A	103 ± 2.65	N/A	N/A
	Treatment	107.50 ± 1.73	107.50 ± 2.38	105.75 ± 7.85	0.84
	Washout	108.25 ± 3.20	106.50 ± 3.87	109.50 ± 1.29	0.40
Anion Gap	Baseline	6.75 ± 2.22	9.50 ± 7.19	8.25 ± 2.63	0.70
	Induction	N/A	10.00 ± 2.00	N/A	N/A
	Treatment	5.75 ± 1.71	8.00 ± 1.83	7.50 ± 1.73	0.21
	Washout	10.00 ± 1.41	10.00 ± 2.94	10.75 ± 2.99	0.89
Phosphate	Baseline	0.72 ± 0.17	0.84 ± 0.35	0.76 ± 0.22	0.79
	Induction	N/A	1.05 ± 0.19	N/A	N/A
	Treatment	0.81 ± 0.11	0.82 ± 0.22	0.84 ± 0.10	0.97
	Washout	0.83 ± 0.18	0.80 ± 0.48	0.65 ± 0.20	0.70
Potassium	Baseline	3.23 ±0.24	3.25±0.30	3.43 ± 0.15	0.46
	Induction	N/A	3.03 ± 0.25	N/A	N/A

	Treatment	4.03 ± 0.41	3.75 ± 0.13	3.48 ± 0.29	0.08
	Washout	2.95 ±0.26	3.43 ±0.50	3.28 ± 0.61	0.39
Bicarbonate	Baseline	33.18 ± 1.62	29.50 ±4.51	31.03 ± 2.26	0.28
	Induction	N/A	36.60 ± 2.43	N/A	N/A
	Treatment	32.20 ± 2.14	32.78 ± 1.63	32.65 ± 1.85	0.90
	Washout	30.08 ± 4.21	29.35 ± 4.59	29.43 ± 4.03	0.96

N/A = Not applicable: the control and spontaneous group were not induced, therefore not comparable.
N/D = Incomplete blood analysis.

Table 2A: Haematology analysis during baseline, induction, treatment and washout

Parameter	Time point	Control	Induced	Spontaneous	P-value
RBC	Baseline	5.77 ± 0.76	5.69 ± 0.20	5.51 ± 0.34	0.75
	Induction	N/A	5.57 ± 0.64	N/A	N/A
	Treatment	5.88 ± 0.90	5.66 ± 0.26	5.28 ± 0.24	0.36
	Washout	6.03 ± 1.08	5.73 ± 0.32	5.55 ± 0.28	0.61
WBC	Baseline	3.65 ± 0.26	5.6 ± 2.41	4.8 ± 1.37	0.27
	Induction	N/A	4.43 ± 0.42	N/A	N/A
	Treatment	4.05 ± 0.83	6.25 ± 3.82	6.175 ± 2.00	0.41
	Washout	3.88 ± 0.41	7.4 ± 3.81	6.725 ± 3.62	0.27
MCV	Baseline	72.75 ± 0.96	71.75 ± 4.11	78.00 ± 3.83	0.05
	Induction	N/A	71.33 ± 4.16	N/A	N/A
	Treatment	73.75 ± 1.50	72.75 ± 3.40	76.75 ± 2.87	0.15
	Washout	73.75 ± 1.50	72.50 ± 3.51	77.50 ± 3.87	0.11
MCH	Baseline	24.00 ± 0.00	23.25 ± 0.96	25.50 ± 1.29	0.02
	Induction	N/A	22.67 ± 2.08	N/A	N/A
	Treatment	24.50 ± 0.58	23.75 ± 1.26	25.25 ± 0.96	0.14
	Washout	23.75 ± 0.50	23.50 ± 1.29	25.25 ± 0.96	0.06
MCHC	Baseline	33.00 ± 0.00	32.50 ± 1.00	32.50 ± 0.58	0.49
	Induction	N/A	31.67 ± 0.58	N/A	N/A
	Treatment	33.00 ± 0.00	32.00 ± 0.82	32.50 ± 0.58	0.10
	Washout	32.50 ± 0.58	32.25 ± 0.96	32.25 ± 0.50	0.84
HCT	Baseline	0.42 ± 0.06	0.41 ± 0.03	0.43 ± 0.04	0.77
	Induction	N/A	0.40 ± 0.07	N/A	N/A
	Treatment	0.44 ± 0.07	0.41 ± 0.03	0.41 ± 0.02	0.68
	Washout	0.44 ± 0.09	0.42 ± 0.04	0.43 ± 0.04	0.80
RDW	Baseline	14.43 ± 0.72	15.40 ± 0.43	15.05 ± 0.66	0.13
	Induction	N/A	16.13 ± 1.10	N/A	N/A
	Treatment	14.35 ± 0.99	15.30 ± 0.57	14.85 ± 1.44	0.47
	Washout	14.28 ± 0.67	15.05 ± 0.62	15.45 ± 0.97	0.14
Hb	Baseline	13.83 ± 1.74	13.20 ± 0.82	13.88 ± 1.28	0.73
	Induction	N/A	12.63 ± 2.35	N/A	N/A
	Treatment	14.20 ± 2.31	13.23 ± 0.86	13.23 ± 0.88	0.59
	Washout	14.35 ± 2.47	13.38 ± 1.30	13.875 ± 1.14	0.73
Neutrophils	Baseline	1.77 ± 0.53	3.21 ± 1.64	2.88 ± 0.99	0.23
	Induction	N/A	2.32 ± 1.11	N/A	N/A/
	Treatment	2.38 ± 1.06	5.05 ± 4.14	4.20 ± 1.88	0.39
	Washout	1.97 ± 0.26	5.63 ± 3.94	5.00 ± 3.48	0.24

Parameter	Time point	Control	Induced	Spontaneous	P-value
Basophils	Baseline	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.32
	Induction	N/A	0.01 ± 0.00	N/A	N/A
	Treatment	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.40
	Washout	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	ND
Lymphocyte	Baseline	1.68 ± 0.34	2.05 ± 0.63	1.70 ± 0.41	0.50
	Induction	N/A	1.88 ± 1.08	N/A	N/A
	Treatment	1.50 ± 0.41	1.00 ± 0.41	1.56 ± 0.52	0.20
	Washout	1.60 ± 0.34	1.45 ± 0.54	1.20 ± 0.14	0.36
Monocytes	Baseline	0.20 ± 0.08	0.35 ± 0.50	0.18 ± 0.05	0.68
	Induction	N/A	0.20 ± 0.20	N/A	N/A
	Treatment	0.18 ± 0.10	0.20 ± 0.00	0.38 ± 0.15	0.08
	Washout	0.28 ± 0.10	0.33 ± 0.21	0.48 ± 0.24	0.34
Platelet count	Baseline	249.25 ± 32.62	220.25 ± 41.45	204.00 ± 64.41	0.43
	Induction	N/A	260.00 ±33.42	N/A	N/A
	Treatment	250.25 ± 15.17	227.75 ± 13.94	213.75 ± 61.66	0.41
	Washout	254.00 ± 35.62	217.75 ± 24.12	225.50 ± 75.67	0.58

N/A = Not applicable: the control and spontaneous group were not induced, therefore not comparable.
N/D = Incomplete blood analysis.

Table A6: Glycine analysis for CSF and plasma at baseline to washout period.

Groups	Animal ID	Baseline		Valproate		Treatment		Washout	
		CSF (µmol/L)	Plasma (µmol/L)	CSF (µmol/L)	Plasma (µmol/L)	CSF (µmol/L)	Plasma (µmol/L)	CSF (µmol/L)	Plasma (µmol/L)
1. Control	399	7.8	455	-	-	8.0	457	8.1	455
	127	9.1	460	-	-	8.9	465	9.3	462
	145	8.9	470	-	-	9.1	477	9.0	468
	178	8.4	528	-	-	8.3	527	8.3	528.5
	Mean	**8.55**	**478.25**			**8.58**	**481.50**	**8.68**	**478.38**
	SD	**0.58**	**33.75**			**0.51**	**31.43**	**0.57**	**33.84**
2. Induced	338	7.5	589	6.6	586	6.6	288	8.4	367
	104	7.1	785	6.1	599	8.2	462	7.5	345
	113	7.2	246	8.3	383	6.9	332	7.8	555
	438	8.5	486	8	465	7.9	245	8.1	271
	Mean	**7.58**	**526.5**	**7.25**	**508.25**	**7.4**	**331.75**	**7.95**	**384.5**
	SD	**0.64**	**224.39**	**1.07**	**103.02**	**0.77**	**93.82**	**0.39**	**120.86**
3. Spontaneous	398	12.7	532	-	-	8.3	292	8.5	300
	409	10.6	498	-	-	9.4	359	12.1	270
	412	11.02	605	-	-	9.3	356	11.2	467
	416	11.5	579	-	-	6.7	274	12	579
	Mean	**11.46**	**553.5**			**8.43**	**320.25**	**10.95**	**404**
	SD	**0.91**	**47.77**			**1.25**	**43.65**	**1.68**	**145.33**

2. LOG SHEET SAMPLE

Check list: behaviour

0 = absent, not observed, not displayed + = weak, poor, minimal

++ = normal, average, moderate +++ = excessive, strong

Date	Exp. No.	Monkey No.	alert	fearful	aggressive	confused	depressed

Check list: motor function and activity

0 = absent, not observed, not displayed + = weak, poor, minimal

++ = normal, average, moderate +++ = excessive, strong

Date	Exp. No.	posture	coordination	locomotion	active	Use of exercise cage

Check list: physical

0 = absent, not observed, not displayed + = weak, poor, minimal

++ = normal, average, moderate +++ = excessive, strong, copious

disc. = discoloured d = discharge s = soft w = watery

Date	Exp. No.	Monkey No.	coat	feces	urine	eyes	nose	ears	genitals	rectal

APPENDIX B: Molecular biology

1. Materials used in this study

MATERIALS	SUPPLIERS
100bp DNA ladder	BiotechBiocom
Agarose	Whitehead Scientific
Boric acid	Merck
Dextromethorphan	Norpham
EDTA	BDH
EDTA blood collection tubes	Pathcare
Ethanol	BDH
Ethidium bromide	Promega
High capacity cDNA reverse transcriptase	Applied Biosystems
Ketamine hydrochloride	Norpham
Oligonucleotides/primers	University of Cape Town
Paxgene Blood collection tubes	Whitehead Scientific
Paxgene Blood collection tubes	Whitehead Scientific
PCR Master mix	Promega
RNA 6000 Nano-kit	Anatech
RNAse free water	Whitehead Scientific
RNAse inhibitor	Applied Biosystem

RNA ladder	Anatech
RNAse ZAP	Applied Biosystem
RT^2 qPCR primer assays	Applied Biosystems
RT^2 SYBR Green Rox PCR Master Mix kit	Applied Biosystems
Sequencing Ready Reaction Kit	Applied Biosystems
Sodium benzoate	Sigma
Tris-base	Sigma
TURBO DNAse kit	Applied Biosystems
Valproate	Sigma
Wizard SV Gel and PCR Clean-Up System	Anatech
Whole blood separation kit	Separations

2. Extraction of DNA from whole blood

Five hundred microliter (500 µl) of buffer AP1 was added into a 1.5ml microfuge tube. A 200-250 µl of anti-coagulated blood was added. The cap of the microfuge tube was closed and mixed by vortexing at top speed for 10 seconds. Buffer AP2 (100 µl) was added and mixed by vortexing at top speed for 10 seconds. The mixture was centrifuged at 12,000 x g for 10 minutes at ambient temperature to pellet cellular debris. A miniprep column was placed into a 2ml Microfuge tube. The clarified supernatant that was obtained from centrigation was pipette into the Miniprep column and centrifuged at 6, 000 X g for 1 minute. The filtrate from the 2ml microfuge tube was discarded. The miniprep was placed back into the 2ml microfuge tube. Buffer W1A (700 µl) was pipette into the miniprep column and allowed to stand at room temperature for 2 minutes and centrifuged at 6,000 X g for 1 minutes. The filtrate from the 2ml microfuge tube was discarded. The miniprep

was placed back into the 2ml microfuge tube. Buffer W2 (800 µl) was pipette into the miniprep column and allowed to stand at room temperature for 2 minutes and centrifuged at 12,000 X g for 1 minutes. The filtrate from the 2ml microfuge tube was discarded. The miniprep was placed back into the 2ml microfuge tube. Buffer W2 (500 µl) was pipette into the miniprep column and allowed to stand at room temperature for 2 minutes and centrifuged at 12,000 X g for 1 minutes. The filtrate from the 2ml microfuge tube was discarded. The miniprep was placed back into the 2ml microfuge tube and centrifuged at 12,000 X g for 1 minute. The miniprep column was placed into a 1.5ml microfuge tube. Buffer TE (80-200 µl) was added and allowed to stand at room temperature for 1 minute. The mixture was then centrifuged at 12, 000 X g for 1 minute to elute genomic DNA.

3. Primers for genotyping

Table B1: The list of designed primers based on human and rhesus macaque sequences.

Gene	Exon	Forward primer sequence	Reverse primer sequence	Product size (bp)	Annealing (°C)
UGT1A6	1	GCAAGGGAGAGGTAGACAGG	AACCCACCAACCTCAGACAG	994	61
	2	GGCTCTAGGAATTTG AAGCCTAC	AGCTGGAAGTCTGGGGTTAG	207	55
	3	GCCTTCATAGTTGCTGAGCC	CAGCATGGGTGATAAAGGCG	115	55
	4	GTGTCTGGCTGTGAAACTCA	CATGAATGCCATGGCCAAAGT	330	55
	5	CCACAGGTGTTCCAGGCATAA	AAAGCACTCTGGGGCTGATT	474	55
UGT1A9	1	GTTCCCTGATGACTCGCACA	AGGAGAGAGGACTTACCATAGGC	879	61
SLC6A9	2	CACTACCTGTCTGGAGAGCC	ACAGTCTGGCCTCTGTACTC	207	58
	3	ATGGAGCACAAATGCCTTGC	CCCTAGTGGGTGGGCTCTAC	263	58
	4	TTGCTGTTGGGTTCTGAGCA	GGTCAGCATCAGGGCTCTAC	344	58.4
	5	TTAACCTCACCTCCTCCTGCT	AGCAGGCACCCTACTTTGTCT	424	58
	6	GGCCCCTAAAGTAGCTCCCA	CACACACATGACCCAGGTAGG	353	63
	7/8	GGTGGTGATTAGGGAGCCAG	CTCAAGATCAGGAGGGGAAAGG	535	59
	9/10	CCGAAATTGGGGAGAGGGAA	CATGTCCTGGCAACTCTGAGC	558	59
	10/11	TGGGCACTCAGGTATGAGGT	GGCCAGGGAACTCACGAAGA	649	59
	12	TGCAAGGAGAGCTGGGATAC	GCGGCTTGTAGGTCCGAG	377	59

4. 5X TBE buffer

54g TBE

27.5 Boric acid

3.7422 EDTA

Add distilled water to a total volume of 1000ml.

5. 1X TBE buffer

100ml of 5X TBE was mixed with 400ml of distilled water to make a total volume of 500ml. this mixture was used to make and run agarose gel.

6. Preparation of agarose gel (1 or 2%)

One gram (1g) or 1.5g of agarose gel was weighed. Fifty millilitres (50ml or 75ml) of TBE working stock solution was added and mixed properly in a volumetric flask. The mixture was heated using microwave for 2minutes to dissolve the agarose. The mixture was cooled at room temperature. Ethidium bromide (1 or 2µl) was added to the mixture and swirled to mix. The combs were inserted into the gel tank properly. The gel was poured slowly into the tank. Using a disposable tip, bubbles were pushed away to the sides. The gel was left to set for 15 minutes or preferably 30min. The first lane of the gel was loaded with the marker. 10µl of prepared sample was pipette out and mixed with 2 µl of 6x loading dye. The mixture was then loaded into each well. After loading all the samples, the gel tank was closed. The power- source was switched on and the gel was runned at 100V/cm for 60 minutes.

7. Tracking Dye III (Maniatis *et al*, 1993)

0.25% bromophenol blue

0.25% xylene cyanol FF

30% glycerol in ddH_2O

Store at 4^oC

8. Protocol for purification of PCR product

Following electrophoresis, the desired band was cut out of the electrophoresis gel and its weight was determined. Membrane Binding Solution (10 µl) was added for every 10 mg of the gel slice. The mixture was vortexed and incubated at 50- 65°C until the gel was dissolved. An equal volume of membrane binding solution was added to the PCR product. The SV Minicolumn was inserted into collection tube. A dissolved gel mixture or prepared PCR product was transferred to the Minicolumn assembly. The mixture was incubated at room temperature for 1 minute. The mixture was centrifuged at 16, 000 X g for 1 minute. A flow through was discarded and Minicolumn was inserted into collection tube. Membranes wash solution (700 µl) and centrifuged at 1 minute. Flow through was discarded and Minicolumn was reinserted into collection tube. The washing step was repeated with 500 µl membrane wash solution. The mixture was centrifuged at 16, 000 X g for 5 minutes. The collection tube was emptied and the column assembly was recentrifuged for 1 minute with the microcentrifuge lid open (off0 to allow evaporation of any residual ethanol. Minicolumn was carefully transferred to a clean 1.5 ml microcentrifuge tube and 50 µl of nuclease free water was added to the Minicolumn. The mixture was incubated at room temperature for 1 minute and centrifuged at 16, 000 X g for 1 minute. The Minicolumn was discarded and DNA was stored at 4°C or -20°C.

9. Sequencing reaction for purified product

Table B2: Sequencing reactions, as described in the Perkin-Elmer ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit manual (1998).

Reagent	**Quantity**
Terminator Ready Reaction mix (dye terminators, dUTP*, dCTP, dATP, dITP**, AmpliTaq DNA polymerase, r*Tth* pyrophoshatase, magnesium chloride, buffer)	8 µl
DNA template	100 - 200 ng
-21 M13 Primer (forward)	1.6 pmol
$dddH_2O$	x
Total volume	10 µl

* dUTP is used in place of dTTP as it results in a better T patterns because dUTP improves incorporation of T terminators.** dITP is used in place of dGTP to minimise band compressions

Table B3: Sequencing reaction using a Hybaid PCR Sprint Thermal Cycler

	Temperature	**Time**
Denaturing	96°C	10 secs
Annealing	50°C	5 secs
Extension	60°C	4 min
Repeat this sequence for 25 cycles, then store at 4°C before purification		

10. Gene expression

Table B4: Ct values for *PGK1*, *GAPDH*, *GLDC*, *AMT* and *GCNT2*

			PGK1		
Group	**Animal ID**	**Baseline**	**Valproate**	**Treatment**	**Washout**
1.Control	399	-		22.59	20.02
	127	20.89		20.37	22.92
	145	20.35		20.51	21.65
	178	16.8		20.78	21.35
2.Induced	338	21.02	20.36	20.43	21.88
	104	20.07	19.74	20.18	20.50
	113	13.92	20.49	18.14	21.40
	438	20.38	20.89	20.90	21.52
3. Spontaneous	398	-		20.18	21.47
	409	20.06		19.94	19.53
	412	-		20.95	20.82
	416	20.49		20.33	20.62
			GAPDH		
Group	**Animal ID**	**Baseline**	**Valproate**	**Treatment**	**Washout**
1.Control	399	-		27.25	22.82
	127	26.45		24.36	25.49
	145	26.76		24.85	24.15
	178	26.37		24.59	22.99
2.Induced	338	25.53	25.02	24.87	24.08
	104	25.93	24.48	24.98	22.52
	113	25.46	25.17	25.49	23.73
	438	26.40	26.49	22.06	22.55
3. Spontaneous	398	-		25.08	24.00
	409	26.20		24.41	22.01
	412	-		25.39	23.52
	416	25.84		24.34	22.54
			GLDC		
Group	**Animal ID**	**Baseline**	**Valproate**	**Treatment**	**Washout**
1.Control	399	-		35.79	33.44
	127	35.31		34.39	34.31
	145	33.18		33.66	34.57
	178	34.75		34.00	34.01
2.Induced	338	33.80	33.82	34.11	32.99
	104	34.59	33.8	33.79	34.21
	113	33.98	33.29	33.89	34.13
	438	34.42	35.37	34.70	34.64
3. Spontaneous	398	-		33.33	34.90
	409	33.01		33.40	33.12
	412	-		33.86	34.18
	416	33.46		33.99	34.13

			AMT		
Group	**Animal ID**	**Baseline**	**Valproate**	**Treatment**	**Washout**
1.Control	399	-	-	26.72	24.96
	127	25.54	-	24.33	25.32
	145	21.61	-	24.92	24.86
	178	19.73	-	24.91	25.06
2.Induced	338	24.93	24.47	24.21	25.16
	104	23.98	23.65	24.11	24.36
	113	18.34	24.39	24.85	24.75
	438	20.85	25.09	25.38	25.23
3. Spontaneous	398	-	-	24.47	25.85
	409	23.43	-	24.91	24.32
	412	33.07	-	24.93	25.04
	416	24.73	-	23.03	24.69
			GCNT2		
Group	**Animal ID**	**Baseline**	**Valproate**	**Treatment**	**Washout**
1.Control	399	-	-	32.96	30.79
	127	30.22	-	29.98	31.72
	145	30.84	-	30.31	31.37
	178	31.04	-	32.07	32.23
2.Induced	338	30.34	29.85	31.02	31.90
	104	30.11	28.50	30.22	30.07
	113	29.62	30.48	30.37	29.67
	438	30.21	31.68	32.11	32.02
3. Spontaneous	398	-	-	30.04	30.23
	409	30.57	-	30.54	29.97
	412	-	-	31.95	33.13
	416	30.78	-	30.52	31.61

Table B5: RNA integrity

Group	**Animal ID**	**Baseline**	**Induction**	**Treatment**	**Washout**
1.Control	399	-		7.10	9.30
	127	8.30		8.00	9.60
	145	8.40		7.80	9.30
	178	7.90		8.50	9.40
2.Induced	338	8.30	8.60	8.50	9.30
	104	8.30	8.50	8.00	9.50
	113	7.80	8.20	8.00	9.40
	438	8.10	8.10	7.80	8.30
3. Spontaneous	398	-		8.10	9.40
	409	7.90		7.90	9.10
	412	-		8.30	9.30
	416	8.80		8.00	9.20

Table B5: Amino acids

Amino acid	Abbreviations	
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	E
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	V	V