Postmortem Toxicogenetics: determining the suitability of blood samples collected for routine toxicological analyses for use in subsequent genetic analyses

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By

Loyiso Abongile Marvin Vuko

Student Number: VKXLOY001

Supervisor : **Bronwen Davies**

Co-supervisors: **Laura Heathfield**

Kathrina Auckloo

Division of Forensic Medicine and Toxicology

Department of Pathology

University of Cape Town

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DECLARATION

I, **Loyiso Abongile Marvin Vuko**, hereby declare that the work on which this dissertation is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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LIST OF ABBREVIATIONS AND SYMBOLS

%	: Percentage
˚C	: Degrees Celsius
A/C/G/T	: Adenine/Cytosine/Guanine/Thymine
A_{230}	: Absorbance at 230 nm
A_{260}	: Absorbance at 260 nm
A260/230	: Ratio of absorbance at 260 nm to absorbance at 230 nm
A260/280	: Ratio of absorbance at 260 nm to absorbance at 280 nm
A_{280}	: Absorbance at 280 nm
ADR	: Adverse drug reaction
ATP	: Adenosine triphosphate
bp	: Base pairs
Ca^{2+}	: Calcium
CYP	: Cytochrome P450
DNA	: Deoxyribonucleic acid
dNTP	: Deoxynucleotide triphosphates
DST	: Department of Science and Technology
EDTA	: Ethylenediamine tetra-acetic acid
EM	: Extensive metaboliser
g	: gram(s)
G	: Grey
GC content	: Guanine-Cytosine content
GHB	: Gammahydroxybutyrate
HREC	: Human Research Ethics Committee
IM	: Intermediate metaboliser
$C_2K_2O_4$	: Potassium oxalate
L	: Liter(s)
LC-MS/MS	: Liquid chromatography-tandem mass spectrometry
m	: Milli
M	: Molar
MBG	: Molecular biology grade
MDA	: Methylenedioxyamphetamine

MDEA : Methylenedioxyethylamphetamine

MDMA : Methylenedioxymethamphetamine

Mg^{2+} : Magnesium

$MgCl_2$: Magnesium chloride

MM : Molecular weight size marker

n : Nano

NaF : Sodium fluoride

NF : Nuclear factor

No-Tox : Samples not handled in a toxicology environment

NTC : No-template control

p : Probability value

P : Purple

PCR : Polymerase chain reaction

PM : Poor metaboliser

PMI : Postmortem interval

PMR : Postmortem redistribution

R : Red

RM : Rapid method

RNA : Ribonucleic acid

SACENDU : South African Community Epidemiology Network on Drug Use

SNP : Single nucleotide polymorphism

SUD : Sudden unexpected death

TBE : Tris-Borate-EDTA

Tm : Melting temperature

Tox : Samples handled in a toxicology environment

UCT : University of Cape Town

UM : Ultra-rapid metaboliser

w/v : Weight/Volume percentage concentration

α : Level of significance

µ : Micro

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ABSTRACT

South Africa has one of the highest prevalences of drug misuse and abuse in Africa. Salt River Mortuary (Cape Town, South Africa), along with other national Forensic Pathology Service providers, receives many cases of suspected drug-related deaths. In some cases, the traditional autopsy – when viewed together with the decedent's history – is not able to indicate whether a drug-related death is accidental or suicidal in relation to altered drug metabolism. Literature has shown that this can be investigated by sequencing gene(s) encoding the implicated metabolising enzyme(s) in a postmortem genetic analysis. However, as such an analysis would normally be performed following the obtainment of postmortem toxicological results, it is imperative to investigate whether blood samples retrieved back from a toxicology laboratory would be sufficient for the said genetic analysis, despite the handling involved in the process of toxicological investigation. To this end, blood samples from 30 deceased individuals in which drug use/abuse may have contributed to death, were collected into two red-top tubes (plain), two grey-top tubes (containing sodium fluoride and potassium oxalate) and one EDTA-containing purple-top tube (control). DNA was immediately extracted from one of each colour tube, while the duplicate red-top and grey-top tubes first underwent a process of toxicological analyses, and then underwent DNA extraction. The concentration, degradation, purity, contamination, and quality of DNA were assessed using real-time PCR, spectrophotometry, forensic DNA profiling, and Sanger sequencing. In contrast to the grey-top tubes, the results showed that the red-top tubes were most suitable for the aforementioned genetic analysis. Overall, the study not only demonstrated that postmortem genetic analysis using samples retrieved from a toxicology laboratory is possible in the local context, but also provided guidelines around the pre-analytical phase of the analysis. These results illustrate the opportunity to investigate these toxicogenetic avenues further, particularly in future expansion of services currently provided at Salt River Mortuary, which may provide families more information about circumstances of their relative’s death.

CHAPTER 1: INTRODUCTION

1.1. Background

Forensic toxicology is a branch of forensic science that studies the adverse effects caused by drugs and chemicals in humans (Lappas and Lappas, 2016). The need for the complete establishment of the field of forensic toxicology in South Africa is reinforced by the high rate of drug use seen in the country. Otu (2011) described South Africa and Nigeria as being "in a class of their own" when it comes to the prevalence of drug use (Otu, 2011). Of all the sub-Saharan African countries, South Africa is considered to have the largest market for illicit drugs (Peltzer *et al.*, 2010). This has been ascribed to many factors, including the movement of large numbers of people across the South African borders (legally and illegally), an increase in the number of international flights into the country since 1994, visa requirements that are relaxed for South Africans travelling across the borders, as well as customs and borders that are not patrolled properly (Peltzer *et al.*, 2010). The prevalence of drug use in South Africa is also suspected to be related to the affluence of the country relative to other African countries, which is seen to facilitate the creation of urban centres, the lifestyles of which are characterised by the rife use of drugs (Peltzer *et al.*, 2010).

The argument about urbanised areas being most affected the most by the rampant use of drugs is supported by the statistics of drug misuse reported within the borders of South Africa. According to data produced by the South African Community Epidemiology Network on Drug Use (SACENDU) for the year 2017, the province of the Western Cape has the second highest prevalence of drug use, after the Gauteng province (SACENDU, 2017); the two provinces in which the biggest cities in the country are found.

The South African government has passed laws aimed at reducing the demand and supply of drugs, including the Drugs and Drug Trafficking Act No. 140 of 1992 and the Prevention and Treatment for Substance Abuse Act No. 70 of 2008 (Pienaar and Savic, 2016). However, the Western Cape-based Salt River Mortuary (along with other mortuaries in the country) still receives cases of drug-related death. There is, however, a possibility that some of those cases do not involve the abuse of the detected drugs, as studies have shown that some people are genetically inclined to experience toxicity (sometimes fatally) from an intake of substances at

doses that are normally not associated with toxicity (Zanger and Schwab, 2013). One way in which such deaths could be further investigated is through a genetic analysis aimed at assessing the sequence of the genes encoding the enzymes that catalyse the biotransformation of the implicated drugs; a service currently not offered by the South African forensic government services. One such group of enzymes is the cytochrome P450 enzyme family, for which an overview is provided below. This chapter also reviews the literature available on the pre-analytical processing that requires consideration before the implementation of the said genetic analysis. Finally, the literature review assesses the storage of blood specimens in blood collection tubes intended for toxicological analysis, within toxicological environments, and prior to the performance of the aforementioned genetic analysis – which is the focus of this study.

1.2. Cytochrome P450 enzyme family

1.2.1. Introduction to Cytochrome P450

The metabolic systems of the human body carry out various chemical reactions to sustain life, including responding to internal stimuli, deriving and processing nutrients from ingested foods, and adequately responding to xenobiotic substances such as drugs and toxic compounds introduced into the body by a variety of mechanisms (Motulsky, 1957). Cytochrome P450 (CYP) is a superfamily of enzymes that catalyses 90% of phase I of metabolic processes (Arici and Ozhan, 2017), which occur largely in the liver (Zanger and Schwab, 2013). Phase I enzymes alter substrates using reactions such as oxidation, hydrolysis, reduction, alkylation, and dealkylation, while phase II reactions include conjugation of products of phase I reactions with glucuronic acid, glycine, acetate, and sulphate to promote elimination (Sono *et al.*, 1996).

CYP P450 enzymes are so called because they are hemoproteins (represented by "cytochrome") and their spectral property allows them to absorb pigment ("P") or light at a wavelength of 450 nanometers (Jaiswal *et al.*, 1985). All members of the CYP group are called monooxygenases due to their mechanism of action (Bernhardt, 2006). This involves the incorporation of a molecular oxygen into the substrate while simultaneously reducing the second molecular oxygen of the disintegrated dioxygen molecule with two hydrogen atoms to form a water molecule (Bernhardt, 2006). However, these enzymes differ in their primary

structure, which reflects their genetic makeup, and forms the basis by which they are differentiated (Nelson, 2004).

In humans, there are 57 putatively functional genes (Nelson, 2018) and 58 pseudogenes encoding CYP enzymes, which are grouped into 18 families and 44 subfamilies according to their sequence similarities (Nelson, 2004). Most of the genes encoding CYP enzymes code for constitutive enzymes that facilitate endogenous non-metabolic processes. Only a few CYP enzymes, belonging to the CYP families 1, 2 and 3, catalyse the oxidative biotransformation of xenobiotic compounds (such as drugs) that are of particular relevance to the topic at hand (Nebert and Russell, 2002).

1.2.2. Family CYP 2

The *CYP 2* family consists of 16 full-length genes that encode several hepatic enzymes that catalyse some of the most important drug-metabolising reactions in humans (Guengerich and Cheng, 2011). One such member of this family is *CYP2D6* (Nebert and Russell, 2002), which will henceforth be the focus of the discussion in this text.

The CYP2D6 enzyme uses hydroxylation and demethylation to metabolise more than 25% of clinically important drugs, including antidepressants, antipsychotics, analgesics, antitussives, b-blocking agents, antiarrhythmics, and antiemetics (Gopisankar, 2017). Moreover, the enzyme plays a pivotal role in the metabolism of methamphetamine (Lin, 1997) – the primary drug of abuse in the Western Cape (where the current study took place) (SACENDU, 2017). There has not been a full characterisation of all the allelic variants of *CYP2D6* (over approximately one hundred); however, it is known that haplotypes (alleles with a set of linked single nucleotide polymorphisms (SNPs)) such as *CYP2D6*1*, *CYP2D6*2* and *CYP2D6*35* cause a normal or increased enzyme activity, while *CYP2D6*9, CYP2D6*10, CYP2D6*17, CYP2D6*29,* and *CYP2D6*41* encode enzymes with decreased activity, and *CYP2D6*3, CYP2D6*4, CYP2D6*5,* and *CYP2D6*6* translate to enzymes with no enzyme activity (Sakuyama *et al.*, 2008).

While monogenic variability can be used to explain some of the pharmacokinetic and pharmacodynamic variability that occurs within and between individuals, a number of factors

influence the manner in which different individuals react to drugs, including non-genetic host factors (Zhang *et al.*, 2011) and epigenetic factors (Zanger and Schwab, 2013). The focus of this study is, however, on the genetic factors.

1.2.3. Genetic influence on CYP activity

The metabolic phenotypes of many allelic variants of the *CYP* genes were studied and described to form the foundation of pharmacogenetics – a branch of pharmacology concerned with the influence of genetic factors in drug response (Gandhi *et al.*, 2004). Four phenotypic classes were inferred from the genotypic data of these genes and described by Benet *et al.* (1996) as follows: (1) poor metabolisers (PM) carry enzymes that are encoded by homozygous alleles that have a complete lack of function; (2) intermediate metabolisers (IM) carry one allele with a complete lack of function and one allele with a normal function, or homozygous alleles that have a reduced function; (3) extensive metabolisers (EM) carry homozygous alleles with normal function (this is referred to as the "normal phenotype" and represents the majority of the population); and (4) ultrarapid metabolisers (UM) have a mutation that increases enzyme activity (this is usually the duplication of the "normal" gene).

Zanger and Schwab (2013) remarked that the pharmacological impact of these different allelic variants is pronounced with PM and UM phenotypes, by reporting that when a pharmacologically active compound was in the blood, it would remain longer in PM individuals due to reduced or no clearance, resulting in inadvertent drug toxicity, while there was a rapid clearance in UM individuals, resulting in the loss of the pharmacological effect of the drug (Zanger and Schwab, 2013). The converse is true for a prodrug. This unintended response to a drug at a conventional dose – called an adverse drug reaction (ADR) – sometimes contributes to sudden unexpected death (SUD), thus entering the purview of forensic investigations. The term 'forensic toxicogenetics' has been coined for the interdisciplinary use of toxicological and genetic investigations in these cases (Sajantila *et al.*, 2010).

1.3. Forensic toxicogenetics

In some SUD cases the cause of death is reported as "undetermined" after the performance of a traditional autopsy, due to lack of gross pathological evidence. It is also possible that routine

ancillary investigations, such as histology and toxicology are also negative in relation to the determination of the cause death. This has led to the emergence of the concept of molecular autopsies – an expansion of the traditional autopsy to include a genetic examination of the deceased (McElroy *et al.*, 2000). Forensic toxicogenetics, as a form of molecular autopsy, is relevant in the local context, given that the fatality rate due to ADRs in South Africa was estimated to be five to ten times higher than the prevalence observed internationally (Warnich *et al.*, 2011).

Academics and practitioners in the field of forensic pathology hold different views on the need for toxicogenetic investigation in postmortem cases. Most of what is empirically known about forensic toxicogenetics is from case reports that involved a single drug, including oxycodone (Jannetto *et al.*, 2002), tramadol (Levo *et al.*, 2003), citalopram (Holmgren *et al.*, 2004), and amitriptyline (Koski *et al.*, 2006). The general finding from these studies was that the polymorphic nature of the CYP enzyme-encoding genes did not have a significant impact on the toxicity of the aforementioned drugs. A review published by Sajantila *et al.* (2010), however, demonstrated how postmortem genetic analyses may provide important insight into the cause and/or manner of death by citing a number of drug-related death cases. This included a toxicity fatality resulting from administration of prescribed fluoxetine medication in a 9-year-old boy (Sallee *et al.*, 2000) and morphine intoxication in a neonate breastfed by a mother taking codeine medication (Koren *et al.*, 2006). Both of these cases involved reported ADRs that implicate the different metabolic capacities exhibited by different polymorphisms of genes encoding CYP enzymes. Sajantila *et al.* (2010) did concede, however, that further research is still required before forensic toxicogenetic analysis can be integrated into standard forensic practice (Sajantila *et al.*, 2010).

It may be suggested that in the routine forensic setting, the performance of a toxicogenetic analysis would be as a response to an observation of inexplicable toxicological results; that is, when parent and metabolite concentrations observed in the blood (and/or other specimens) of the deceased are inconsistent with medical/circumstantial history, which would warrant a genetic analysis to try and make sense of the observed discrepancy. It is, therefore, important – when designing a strategy on how the test would be applied routinely in situations such as those described above – to review the pre-analytical aspects involved in forensic toxicology

investigations that may affect the ability to perform a subsequent genetic analysis using the same samples.

1.4. The pre-analytical process of postmortem investigations in forensic toxicology

1.4.1. Introduction

The factors that commonly lead to variations in analytical results can either be *in vivo* or *in vitro* (Wisser *et al.*, 2002). Although it is important for analysts to be cognisant of all of these factors, there is little one can do to prevent the contribution of the *in vivo* influences in the variation of analytical results, particularly in postmortem cases where the postmortem interval (PMI) is uncontrolled and usually unknown (Guder, 1999). However, *in vitro* interferences – which include components of sample composition, storage, and processing (Skopp, 2004) – may be controlled, and it is around them that an effective analytical strategy must be devised in order to limit/prevent drug concentration alterations.

Postmortem investigations are, by nature, interdisciplinary. The different forms of training the officers involved (including forensic pathologists, forensic pathology officers, police officers, and toxicologists) receive means that there is no guarantee that the guidelines provided on how to collect different sample types will always be followed. Another way in which the interdisciplinary nature of postmortem investigations manifests its significance in pre-analytical processes is in the selection of an appropriate sample – an exercise that forms part of a broader group of factors in the pre-analytical process collectively known as sample handling (Kerrigan, 2013).

1.4.2. Sample handling in the context of forensic toxicology

1.4.2.1. Specimen selection: significance of blood in postmortem toxicology

Over the years in the field of forensic toxicology, and particularly postmortem toxicology, research has been conducted to identify the biological specimens that are best suited and informative for general and specific drug and chemical analysis (Kerrigan, 2013). As such, the choice of specimen(s) to take for a particular case is directed by both the specific details of that

case and the purpose for the toxicological analysis (Drummer, 2004). Blood is collected more frequently than other biological specimens because of the added benefits it offers over other specimens (Kerrigan, 2013). Urine, vitreous humour, and gastric contents are also collected by forensic pathologists in most drug-death cases. Most alternative specimens such as liver, brain, muscle, fat, and bone tissue, are usually used for more specialised testing (Kerrigan, 2013).

Blood is the most commonly used specimen for analytical purposes in forensic toxicology, particularly in postmortem toxicology, as analytical results are usually utilised to distinguish whether a drug may have contributed to intoxication (Kerrigan, 2013). This can be attributed to the vast amount of research that has focused on blood for this purpose of interpretation in ante- and post-mortem specimens (Wille *et al.*, 2009). However, although postmortem blood is usually available for toxicological analysis, there is variability in drug concentration between different blood collection sites (Dalpe-Scott,1995; Zilga *et al.*, 2017), which explains why it is important to note the exact site from which blood is collected.

The two main types of blood that may be collected at autopsy internationally are central blood (taken from the right chamber of the heart) and peripheral blood (taken from areas distant to the torso region, the most common ones being femoral and iliac) (Kerrigan, 2013). Forensic pathologists are discouraged from taking central blood, as it is considered to be non-homogeneous (Jones, 2007) and more susceptible to contamination and postmortem redistribution (PMR) (Yarema and Becker, 2005). While central blood may be suitable for screening purposes, peripheral blood is recommended for quantitative analysis, as it is suggested that PMR is less so in femoral blood (Kerrigan, 2013). However, this doesn't negate the possibility of distribution in femoral sites between death and autopsy (Gerostamoulos, 2012). Regardless of the site of blood collection, the requirements for proper storage are the same.

1.4.2.2. Specimen storage

Pharmacokinetics involves introduction of xenobiotic compounds into the body, which are acted upon by the body's physiological mechanisms – changing the compound's physicochemical properties in the process – in an effort to remove the foreign material from the body (Benet, 1995). Scientists have established that the conversion of drugs into their

hydrolytic derivatives takes place even after death in processes that can happen both *in situ* and *in vitro* (Drummer, 2004). Several studies have shown how the putrefactive processes that take place after death could significantly alter the concentration of drugs in the body. For instance, Moriya and Hashimoto (2003) demonstrated how anaerobic bacteria – which play a central role in the process of decomposition – may be responsible for the observed conversion of nitrobenzodiazepines such as flunitrazepam, nitrazepam, clonazepam, and nimetazepam, to their respective 7-amino-metabolites (Moriya and Hashimoto, 2003). Minimising microbial activity in the body of the deceased, which can be achieved by creating conditions under which microbial growth is unfavoured, has been recommended. Mortuaries employ low temperature-controlled conditions to limit the putrefactive process and hinder microbial activity. Additives may also be used *in vitro* to hinder this activity, as discussed later on.

When a sample of interest has been isolated from the body of the deceased, this preservative measure can be maximised by subjecting the isolated specimen to temperatures (among other preventative measures) certified to best limit the decomposition of the compound of interest. For instance, El Mahjoub and Staub (2000) found that benzodiazepines in whole blood samples are stable when stored at –20˚C, demonstrating the importance of temperature control in the pre-analytical process of toxicological analysis (El Mahjoub and Staub, 2000). Interestingly, the close relatives of benzodiazepines, 7-amino-benzodiazepines, are considerably unstable at this temperature; stability for this group of drugs when contained in postmortem blood, is reportedly achieved when the matrix is stored at –60˚C (Robertson and Drummer, 1998). This suggests that there is no universal temperature under which all drugs in a biological matrix remain stable for a specific amount of time, largely due to varying physicochemical properties. Stability of drugs in biological matrices in varying containers and at different temperatures are therefore aspects that should be evaluated by the toxicology laboratory.

The evaluation of the suitable temperature for the stability of drugs is, however, of limited importance when considered in isolation; this piece of information can best be utilised in combination with knowledge on the other requirements for the stability of the drug of interest. For example, although designer drugs MDA, MDMA and MDEA are all stable at –20˚C, this state is maintained for a short period in whole blood when compared to other matrices such as serum and urine (Clauwaert *et al.*, 2001), which highlights the relevance of sample selection when considering sample storage.

The importance of insight into the various factors that influence the stability of drugs in postmortem matrices was also demonstrated in a study by Rees *et al.* (2012), wherein the stability of 6-acetylmorphine (a unique heroin metabolite) in different matrices (blood, vitreous humour, and homogenised skeletal muscle) stored with and without a preservative (sodium fluoride) at different temperatures (4˚C and –18˚C), was studied over a period of 84 days. While the compound was more stable – in all matrices – when the temperature was set at –18˚C, the paramount finding of the study was that regardless of the matrix used and the temperature under which the specimens were stored, the presence of sodium fluoride substantially increased the stability of 6-acetylmorphine, which the authors attributed to the preservative's ability to inhibit the activity of microorganisms in a biological specimen (Rees *et al.*, 2012).

The preservative power of sodium fluoride was also demonstrated by Fjeld *et al.* (2012), who performed reanalysis on specimens that were stored at –20˚C for a period of up to 7.2 years and found that the stability of the tested drug (gamma-hydroxybutyrate) had not been affected to a significant degree (Fjeld *et al.*, 2012). One of the most common uses of sodium fluoride as a preservative is in the storage of biological specimens for ethanol analyses. It has been shown that microorganisms such as *Candida albicans* and *Escherichia coli* are able to produce ethanol from glucose (Quintas *et al.*, 2017), which may mislead toxicologists in the interpretation of analytical results. This process has also been shown to be preventable with the addition of sodium fluoride in the specimen of interest, which inhibits the activity of the ethanol-producing microorganisms (Yajima *et al.*, 2006).

The use of sodium fluoride highlights the use of additives as preservatives of drugs in the biological matrix. However, toxicologists also use sample vials with additives intended to preserve the natural state of the matrix, and in turn the drugs of interest as well. This is especially important for complex biological specimens such as blood, the cellular content of which makes it susceptible to postmortem changes. This may be due to the enzymatic, hydrolytic, and oxidative activities that take place as a result of microbial invasion and/or proliferation, as well as the release of endogenous enzymes in the process of necrosis or apoptosis (Alaeddini *et al.*, 2010). An anti-coagulant is used in this case to prevent coagulation and degradation of blood cells. Examples of anticoagulant additives include ethylenediamine tetra-acetic acid (EDTA), potassium oxalate, citrate, and heparin. The use of

these is important in many fields where prevention of blood coagulation is of importance to the scientific analysis to be performed. This includes genetics, where blood is often used as the source of DNA.

1.4.3. Sample handling in the context of genetics

1.4.3.1. Effects of anticoagulants on DNA

The Division of Forensic Medicine and Toxicology in the University of Cape Town consists of a branch that offers forensic pathology investigation services under the Western Cape Provincial Government's Forensic Pathology Service and a scientific branch called Biomedical Forensic Science, whose focus is academic research and development aimed at improving the manner in which forensic services are rendered to the West Metropole of Cape Town. One of the components of the latter branch is Molecular Forensics – the unit under which genetics-based forensic research falls. When using blood as a biological sample for genetic analysis, geneticists make use of EDTA to prevent the coagulation of blood and to render it viable for the extraction of high quality DNA on which subsequent molecular analyses are based. This is in the form of blood collection tubes with a purple lid (this project made use of 4 mL vials supplied by BD Vacutainer (New Jersey, USA) containing 7.2 mg of K_2EDTA) – referred to here as Purple-top tubes.

The efficacy of EDTA in preventing coagulation without adversely affecting the quality and quantity of the DNA in blood was demonstrated by Färne *et al.* (1999). In the study, EDTA was compared to other anticoagulants (including citrate and heparin) and was found to be comparable to citrate in its effect on the detectability of DNA by polymerase chain reaction (PCR) after the treatment of blood with the tested additives (Färne *et al.*, 1999). Heparin was, on the other hand, found to inhibit PCR – an effect that the investigators were able to reverse by adding *heparinase I* in the heparin-treated blood (Färne *et al.*, 1999). This demonstrated an important aspect of the treatment of biological specimens, and how this must suit the context of the intended investigation. While heparin was successfully used to prevent the coagulation of blood, it was rejected as a suitable additive on the basis that it did not allow the investigators to perform the required molecular assay (Färne *et al.*, 1999).

The study conducted by Färne *et al.* (1999) confirmed an observation made by Holodniy *et al.* (1991), who determined the effect of a number of anticoagulants (including acid citrate dextrose, sodium EDTA, potassium oxalate, and sodium heparin) on the application of PCR on the DNA extracted from whole blood, plasma, and separated mononuclear cells that were stored in the presence of the aforementioned anticoagulants (Holodniy *et al.*, 1991). The researchers found that for all the tested biological specimens the signal obtained from the enzyme-linked affinity assay (used to quantify the PCR products) was significantly attenuated for all the samples that were stored with sodium heparin, illustrating the inhibition of the PCR process – a phenomenon that they, too, were able to remedy by adding *heparinase* to the samples stored with sodium heparin (Holodniy *et al.*, 1991). The inhibitory effect of heparin in PCR reactions was again proven in a recent study wherein *heparinase* was used to negate the inhibition of the amplification of microRNA (Li *et al.*, 2016).

1.4.3.2. Effects of preservatives on DNA

This text has explored the role of EDTA as an anticoagulant and the importance of this function in the downstream processing of blood and its contents (DNA). However, Lahiri and Schnabel (1993) showed that the role of EDTA, as far as blood and DNA are concerned, does not end with its anticoagulative effect. In the said study, magnesium chloride ($MgCl_2$) – which was required in a procedural step they employed to extract DNA from blood, termed the modified rapid method (RM) procedure – was found to have a degradative effect on the extracted DNA (Lahiri and Schnabel, 1993). Upon making this observation, EDTA was introduced into the vials in which DNA extraction was performed and successfully inhibited the effect of $MgCl_2$, thus effectively preserving DNA through direct interaction with components of the blood mixture (Lahiri and Schnabel, 1993). This provides an uncommon dimension in the use of EDTA as an additive in storage, where a compound reputable for its anticoagulative properties is employed for preservative purposes.

The successful use of EDTA in this manner can be attributed to the acid's ability to form chelates with metal ions, which are often required as cofactors for enzymes (including endogenous and exogenous *nucleases*). The chelation of these metal ions by EDTA makes them unavailable to the enzymes that require them for their function and thus protecting the substrates of the enzymes (nucleic acid molecules) by rendering the *nucleases* inactive. It was

shown by Huang *et al.* (1997) that Mg^{2+} is required for the production of high molecular weight fragments of DNA, whereas the production of oligonucleosomal fragments requires the presence of the two divalent cations: Mg^{2+} and Ca^{2+} (Nakamura *et al.*, 1981; Sun and Cohen, 1994).

The ability of sodium fluoride to prevent the decomposition of most drugs in a blood matrix was discussed above. However, to the author's knowledge, there are no reports in the literature on what effect this additive has on DNA in postmortem blood. It is widely accepted that excessive exposure to fluoride causes a condition called fluorosis (Vani and Reddy, 2000). This phenomenon could be explained by studies that have shown that fluoride intoxication can render cells energy-deficient by altering mitochondrial functions through the inhibition of protein synthesis and nucleotide damage (Jeng *et al.*, 1998).

The mechanism by which the fluoride ion exerts its destructive effect on DNA is still elusive; however, some explanations have been propounded. Some scientists have suggested that fluoride ion binds specifically with certain riboswitch molecules, which undergo a conformational change upon the addition of a fluoride ion, contributing to the loss of their stability (Ren *et al.*, 2012). Using B-form DNA to investigate the effect of fluoride, Liu *et al.* (2017) also demonstrated that the negatively charged ion affects the stability of DNA hairpin (Liu *et al.*, 2017).

The physiological effect of sodium fluoride on DNA was shown whereby a group of mice was administered sodium fluoride for a period of 30 days while two control groups were given water and black tea (whose anti-oxidative function is known to prevent the destructive effect of sodium fluoride) (Anamika *et al.*, 2012). The livers of the mice administered with sodium fluoride yielded a significantly low amount of DNA when compared to the control groups (Anamika *et al.*, 2012). It should, however, be noted that this was an *in vivo*-based study in that the sodium fluoride was introduced into living organisms and exerted its effect on living cells.

The effect of sodium fluoride in an *in vitro* setting was investigated by Zhanga *et al.* (2008), who tested the fluoride-induced damage of DNA on isolated primary rat hippocampal neurons. In addition to the up-regulation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and the arrest of the S-phase cell-cycle, the authors found that the treatment of

the neurons with sodium fluoride significantly damaged the DNA (Zhanga *et al.*, 2008). One may argue, however, that the morphology of neural cells may prevent us from using the findings of this study to extrapolate on the effect of sodium fluoride on the DNA in a blood matrix. This concern was addressed by a study conducted by Podder *et al.* (2015) in which human blood from healthy adult individuals was used to demonstrate that sodium fluoride was responsible for the observed DNA fragmentation and could be associated with the induction of apoptotic processes in the U87 and K562 cells (Podder *et al.*, 2015).

One notes, however, that none of the cited studies used postmortem blood in their experiments, which has properties that are markedly different to those of antemortem blood, as is seen in the discrepancies in the levels of ATP, some purines and pyrimidines, and myoglobin (El-Seshley *et al.*, 1999). The fragmentation of DNA (in postmortem blood) as a result of the action of internal *nucleases* that are released from cells shortly after death as part of an apoptotic or necrotic process, is another marked difference (Johnson and Ferris, 2002; Nazir *et al.*, 2011).

1.5. Knowledge gap and motivation

While there is evidence to show that toxicogenetic investigations can assist with the determination of the cause and/or manner of death, there has been limited research under this topic in a South African context. It is, therefore, imperative to conduct a pilot study that investigates the need for incorporating toxicogenetic analysis as part of the examinations performed in South African mortuaries. Information gathered from such a study would provide baseline data for the development of further genetic assays targeting genes known to be associated with the pharmacokinetics of drugs in the body, in a South African context. Such an analysis may be incorporated into the autopsy procedure currently in operation at Salt River Mortuary, which requires that the proposed model be compatible with the existing postmortem investigation practices.

According to the Department of Health in the Western Cape Government (2014), Salt River Mortuary is an M6 graded facility that receives over 3500 cases per annum. A caseload of that magnitude coupled with the limitation of national resources available to Western Cape's Forensic Pathology Service dictates that only selected cases can be afforded an investigation that encompasses all of the ancillary forensic examinations that include toxicological tests.

At Salt River Mortuary, only those cases for which some evidence of exposure to drugs, chemicals or other substances is brought to the attention of the assigned pathologist, usually undergo a comprehensive toxicological investigation. In addition, cases of sudden, unexplained death where there are no gross pathological findings at autopsy usually undergo toxicological and histological examinations. In such cases, blood is the biological specimen of choice, owing to its advantages over other biological samples, which include the availability of a great magnitude of literature that can facilitate the interpretation of analytical results, and the numerous collection sites from which to choose (Moffat *et al.*, 2004).

Kerrigan's (2013) review of biological specimens commonly used in forensic toxicology illustrated that some specimens are better suited to certain purposes (screening versus quantitation, or targeted drug identification) than others. However, almost all postmortem specimens have limitations that need to be considered in collection, analyses and interpretation. Some of these specimens are complex matrices, often requiring extensive preparation that is both expensive and time-consuming. This includes tissues like the brain and liver. Some specimens have limited quantitative and therefore interpretive value, and others are prone to postmortem putrefactive effects. While specimens such as urine provide an indication of exposure and have value in the case context, the use of blood – particularly peripheral blood – in routine postmortem toxicological analyses has been adopted globally and locally, given its interpretive value in most cases.

Currently, when the need to perform a toxicological investigation for a case examined at Salt River Mortuary arises, femoral blood samples are collected into glass collection vials. These are either plain (have no additives) (red-top tubes) or contain sodium fluoride and potassium oxalate (grey-top tubes). The literature reviewed has not reported any negative effects of the use of potassium oxalate for blood storage (Holodniy *et al.*,1991); however, it has shown how DNA can be adversely affected by sodium fluoride (Ren *et al.*, 2012; Podder *et al.*, 2015; Liu *et al.* 2017). It should be noted that none of the studies performed have shown what the effect of having both of these compounds in a storage container are on the DNA contained in the blood under storage. The literature review has also highlighted the fact that no researchers have reported on the effect of these compounds on postmortem blood, which has been shown to be considerably different to antemortem blood (El-Seshley *et al.*, 1999; Johnson and Ferris, 2002;

Nazir *et al.*, 2011). The nature of postmortem forensic investigations is such that samples not taken at autopsy usually can't be taken at a later stage when the need for them is realised, as the body would most likely have been released to the family of the deceased. Samples for DNA testing are not taken routinely in the current environment. Since we envisage that toxicogenetic analysis would be performed upon the interpretation of toxicological results, it is crucial to determine if the handling of postmortem blood specimens collected into collection tubes currently employed at Salt River Mortuary for the performance of toxicological analysis would not compromise the quality of the DNA contained in the blood in a way that renders the genetic material unsuitable for sequencing the genes of the target enzymes at a later stage.

The outcome of such an investigation would give an indication as to whether blood samples currently collected at autopsy and handled in a toxicological environment are sufficient for use in the proposed molecular assay or whether such an assay would require the collection of blood samples in collection tubes specifically designed for the collection and storage of blood for the purpose of genetic analysis.

1.6. Aim and objectives

The aim of this study was, therefore, to investigate whether blood samples collected and stored in collection tubes intended for toxicological analysis, were suitable for use in a genetic analysis, following handling involved in the process of toxicological investigation.

To this end, the objectives were:

- Recruitment of 30 postmortem cases identified to have a toxicological relevance at Salt River Mortuary.
- Collection of postmortem blood specimens into three types of sterile blood collection tubes.
- Extraction of endogenous DNA from all the collected blood samples, before and after toxicological analysis.
- Quality assessment of extracted endogenous DNA contained in all of the collected blood samples by various molecular methods.

CHAPTER 2: MATERIALS AND METHODS

2.1. Participant recruitment

The study consisted of a cohort of 30 postmortem cases selected from Salt River Mortuary. A minimum of 30 cases was targeted to confer statistical significance to the outcome of the project. The mortuary is one of 17 in Western Cape, South Africa, and receives cases from the West Metropole of Cape Town. The study obtained ethics approval from the University of Cape Town's Faculty of Health Sciences Human Research Ethics Committee – REF: 110/2017 (Appendix A).

2.1.1. Inclusion criteria

Cases that were deemed relevant to the study were those whose cause of death was known or suspected to involve drug toxicity, which warranted the collection of blood specimens (at autopsy) for toxicological analysis. Cases in which there was a sudden and unexpected death of an adult (SUDA), and for which a history of drug use was reported, were also included in the study. Only those cases for which informed consent from the next-of-kin of the deceased was obtained were included in the study.

2.1.2. Exclusion criteria

Cases wherein the corpse was received in a condition that may have compromised the quality of the DNA, including cases of decomposition and immolation, were not included in the study. All cases in which the decedent was below the age of 18 years were also excluded from the study.

2.1.3. Informed consent

The description of cases received by the mortuary was reviewed daily to select relevant cases. For each case selected, a meeting between the researcher and the next-of-kin of the deceased was arranged. The meeting was conducted in a manner that upheld the declaration of Helsinki (1964) as amended in 2013 by the 64th World Medical Association General Assembly in

Fortaleza, Brazil, and was within the ethical framework previously established in-house (Heathfield *et al.*, 2017). In addition to providing the next-of-kin with an information form (Appendix B) explaining the purpose of the study, the procedure involved, and the risks and benefits of the study, all of the relevant information was communicated verbally to the family members present in the meeting to ensure that they understood all the important aspects of the study. This was performed in the families' language of choice.

Conducting the meeting face-to-face also afforded the family members an opportunity to express the concerns they may have had about participating in the study and provided the researcher with a platform to answer any relevant questions. Having ensured that the family members fully understood the study, informed consent was formally requested from the next-of-kin to collect a specified volume of blood from their deceased family member and to subject it to the experiments of the study as described to the family. Signatures from the next-of-kin and a witness present in the meeting were obtained if agreed upon.

2.2. Sample collection

For those cases for which informed consent was obtained, femoral blood specimens were collected from the deceased. Blood was drawn from the femoral vein of the deceased and transferred into one 4 mL purple-top tube (denoted by P herein) (BD Vacutainer, New Jersey, USA), two 4 mL grey-top tubes (denoted by G herein) (SG Vac), and into two 10 mL red-top tubes (denoted by R herein) (SG Vac). The volume of blood that was aliquoted into each of the five tubes was 4 mL. The grey-top tube (containing potassium oxalate and sodium fluoride) and the red-top tube (with no additives) are equivalent to the blood collection vials used at Salt River Mortuary for samples intended for toxicological investigations. These were, therefore, the primary focus of the study and feature prominently in the discussion of the study results. The purple-top tube (containing EDTA) is the standard tube used at Salt River Mortuary for genetic-based investigations and served as a control vial in the study.

The unique identifier code that was given to each sample was used throughout the study to maintain confidentiality and to track the blood specimen collected into the tube as well as the samples (e.g. extracted stock DNA, diluted DNA sample, etc.) that were derived from it.

2.3. Post-collection processing

Following collection at Salt River Mortuary, all the blood specimens were immediately transported to the University of Cape Town's Molecular Forensics' laboratory. The samples were stored at 4˚C and DNA was extracted within 72 hours of sample collection from all the samples that would not undergo toxicological analysis, identified as the "No-tox" samples: one grey-top tube (G-No-tox), one red-top tube (R-No-tox), and the purple-top tube (P-No-tox) (Figure 2.1).

The remaining grey-top tube and red-top tube (called G-Tox and R-Tox, respectively) remained stored at 4˚C at UCT's Forensic Toxicology Unit laboratory until downstream toxicological analysis was performed (Figure 2.1). These samples were, therefore, called "Tox" samples.

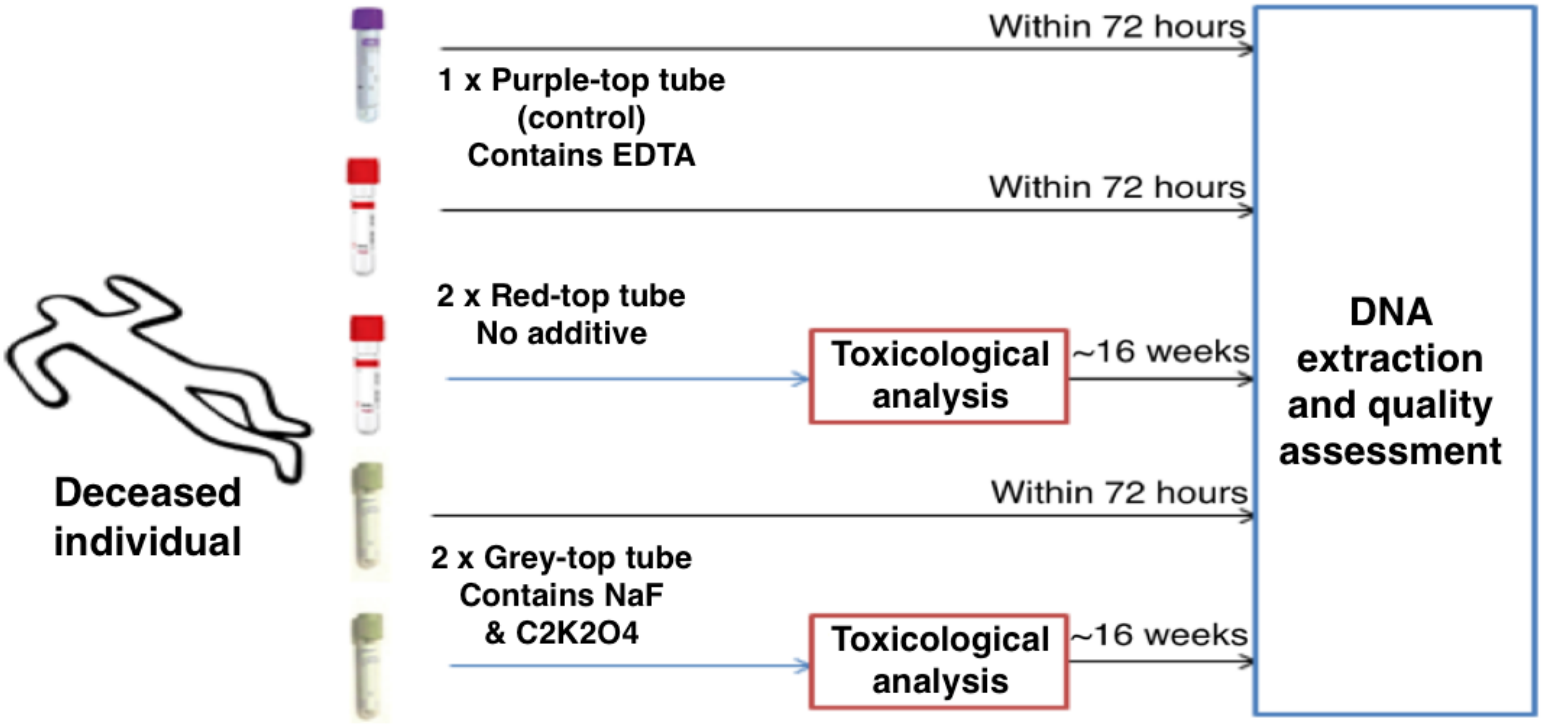


Figure 2.1: **Collection of five blood samples from decedents and overview of downstream processing**. Following the selection of a postmortem case of interest and the recruitment of the participant thereof, five femoral blood samples (4 mL) were collected from the deceased into three types of blood collection tubes: 1 × purple-top tube, 2 × grey-top tubes, and 2 × red-top tubes. The blood samples were stored at 4˚C and DNA was isolated within 72 hours of blood collection from one of each tube type. The remaining grey-top and red-top tubes were handled in a toxicological environment (at 4˚C) and DNA was extracted approximately 16 weeks after blood collection. The QiaAmp DNA Investigator's Kit (Qiagen, Hilden, Germany) was used for DNA extraction.

2.4. Laboratory work

2.4.1. Toxicological processing

In the routine work of Forensic Pathology Service at Salt River Mortuary, forensic pathologists may collect blood samples (together with other specimens) from cases in which drugs and or alcohol are suspected to have caused or contributed to death. A toxicological qualitative analysis is initially performed on these blood specimens to obtain a preliminary screen of targeted drugs of abuse if the pathologist requests. This procedure was simulated in this study. Within 7 days of sample collection, a volume of 500 µL of blood was aliquoted from the G-Tox and R-Tox samples and transferred into a fresh grey-top tube to make up a total volume of 1 mL for each case. Sample preparation in the form of acetonitrile protein precipitation was performed on the aliquots, before they underwent screening by means of liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis using a Shimadzu Prominence High Performance Liquid Chromatography (Tokyo, Japan) system coupled to a AB SCIEX API 3200 Q-TRAP® Mass Spectrometer (Massachusetts, USA) that operates on the AB SCIEX MasterView™ software (Massachusetts, USA). The remainder of the blood in the Tox tubes remained under storage at 4˚C until another aliquot was taken for a simulated quantitative analysis.

Finally, DNA extraction was performed on the Tox-samples, which was approximately 16 weeks after the original blood collection (Figure 2.1).

2.4.2. DNA Extraction

DNA was extracted from all the five blood specimens collected for each case. The QiaAmp DNA Investigator's Kit (Qiagen, Hilden, Germany) was used, with the slight modification of centrifugation at 10 285 × g instead of 6 582 × g in step 9 of the protocol. The process used 100 µL of blood, from which DNA was isolated and eluted into 50 µL of Qiagen ATE Elution Buffer (this was referred to as elution 1). The elution step was repeated into a separate vial with another 50 µL of the Qiagen ATE Elution Buffer to produce elution 2, such that at the end of each DNA extraction procedure there were two 50 µL samples of isolated DNA (elution 1 and elution 2) for each blood sample.

2.4.3. Quality assessment of the extracted DNA

2.4.3.1. Assessment of the quantity and purity of DNA by spectrophotometry

DNA was initially quantified using NanoDrop 2000 (Thermo Scientific, Massachusetts, USA). To determine the concentration of DNA in each sample, a volume of 2 µl of the sample was placed onto the instrument's pedestal and the concentration of DNA in the sample read from the system's software, NanoDrop 2000/2000c Software (Thermo Scientific, Massachusetts, USA).

To determine the purity of the extracted DNA sample, the 260/280 and 260/230 absorbance ratios were noted during each concentration reading. The absorbance ratio of 260/280 provided an indication of the amount of proteins and peptides present in the sample, and the absorbance ratio of 260/230 gave an indication of the extent to which the sample was contaminated with impurities such as phenolic compounds, EDTA, carbohydrates, and Guanidine hydrochloride (Desjardins and Conklin, 2010).

2.4.3.2. Assessment of the quantity and degradation of DNA by qPCR

Following the manufacturer's protocol, qPCR was set up using the Quantifiler™ Trio DNA Quantification Kit (Applied Biosystems, California, USA). The combined Quantifiler™ Trio assays separately amplify a small and a large human autosomal target, as well as a specific region in the human Y chromosome. The ratio of the quantification result of the small autosomal target to that of the large autosomal target – known as the degradation index – was used as an indicator of DNA degradation. The result of the quantification of the Y target was used to confirm if the amplified DNA belonged to a male individual as a quality measure.

As suggested in the manufacturer's user guide, DNA quantification standards were prepared as five serial dilutions separated by a dilution factor of 10. A duplicate of these standards was also prepared. Elution 2 DNA samples were used for this analysis. Molecular biology grade (MBG) water (Lonza, Basel, Switzerland) was used to dilute some of the elution 2 samples

such that they were all below the concentration of 50 ng/µL, which was the upper limit of the calibration curve that could be plotted using the aforementioned DNA quantification standards.

Thermal cycling was performed according to the manufacturer's protocol. All 150 DNA samples (30 purple-top tubes + 60 (30 G-No-Tox + 30 G-Tox) grey-top tubes + 60 (30 R-No-Tox + 30 R-Tox) red-top tubes) that were acquired for this study were analysed by qPCR.

2.4.3.3. Assessment of the contamination of DNA by forensic DNA Profiling

A) Preparation of DNA samples and multiplex PCR amplification

DNA profiling was performed to assess if contamination had been introduced to samples which had been used for toxicological analysis. The PowerPlex® ESI 16 System (Promega, Wisconsin, USA) was used to prepare multiplex PCR according to the manufacturer's technical manual, with the only deviation being that for every reagent, one quarter of the reagent volumes were used to prepare the required master mix. This was in accordance with the manufacturer's approval and internal validation.

Appropriate aliquots of DNA were taken from the stock DNA samples (extracted from the Tox samples only) to prepare DNA dilutions with a concentration of 0.5 ng/µl for each sample. A no-template control (NTC) PCR reaction mixture was also prepared by adding MBG water into the prepared master mix in the place of DNA. The instructions provided in the technical manual were followed to perform PCR on a BioRad (California, USA) T100 thermal cycler system. The multiplex system amplified the following loci: Amelogenin, D3S1358, D19S433, D2S1338, D22S1045, D16S539, D18S51, D1S1656, D10S1248, D2S441, TH01, vWA, D21S11, D12S391, D8S1179, and FGA.

B) Resolution and detection of amplicons by capillary electrophoresis

Following PCR, each sample was added into an Applied Biosystems MicroAmp® optical 96-well plate containing WEN Internal Lane Standard 500 and Hi-Di™ formamide (Promega, Wisconsin, USA) as recommended in the technical manual. The injection time was set at 5 seconds, the injection voltage at 3 kV, and the run time at 1 500 seconds before the samples were run on an Applied Biosystems Genetic Analyser 3130*xl* at 60 ˚C. The results of the

capillary electrophoresis were analysed using the Applied Biosystems GeneMapper v4.1 software.

2.4.3.4. Assessment of the quality of DNA by Sanger sequencing

The overall aim of the study was to test whether blood specimens that are taken to toxicology laboratories for a toxicological analysis could subsequently be used to extract DNA that is of sufficient quality to perform a toxicogenetic analysis. The toxicogenetic analysis of interest in this case is one that examines the sequence of *CYP2D6*, which has nine exons. Primers that amplify these exons were designed in-house in a parallel study. Study case 22 was selected for this experiment and 22G-Tox and 22R-Tox were the DNA samples that were used. (The prefix before the sample type denotes the study case number. This kind of notation is used throughout this text).

A) *Amplification of the exons of CYP2D6 using the designed primers*

Forward and reverse primers were prepared to amplify a target segment of the DNA, and Table 2.1 shows the details of the primers as well as the exons that each of those segments belong to.

Table 2.1: Target exons of *CYP2D6* and the corresponding primers.

Target	*CYP2D6* exon	Primer sequence (5' to 3')	Primer Direction	Length (bp)	GC content (%)	Predicted T*m* (ºC)	Product size (bp)
A	Part of 1	GCCATCATCAGCTCCCTT	Forward	18	55.6	54.9	439
		CCCAAACCTGCTTCCCCTT	Reverse	19	57.9	57.9	
B	Part of 1	CCCTACCAGAAGCAAACA	Forward	18	50.0	52.0	597
		CCTATTTGAACCTTGGACGA	Reverse	20	45.0	52.1	
C	Part of 1	CTTCCACCTGCTCACTCC	Forward	18	61.1	55.3	314
		TCTGTCTCTGTCCCCACC	Reverse	18	61.7	56.1	
D	2 and 3	GTGGATGGTGGGGCTAAT	Forward	18	55.6	54.6	483
		ACTCCTCGGTCTCTCGCT	Reverse	18	61.1	57.7	
E	4	CCCGTTCTGTCTGGTGTAG	Forward	19	57.9	54.9	266
		AGCCTCCCCTCATTCCTC	Reverse	18	61.1	56.3	
F	5 and 6	GTTCTGTCCCGAGTATGC	Forward	18	55.6	52.7	334
		CCTGACACTCCTTCTTGC	Reverse	18	55.6	52.9	
G	7	CATAGGAGGCAAGAAGGAG	Forward	19	52.6	52.1	382
		TGGTGGCATTGAGGACTA	Reverse	18	50.0	53.1	
H	8	ATCCTAGAGTCCAGTCCC	Forward	18	55.6	52.3	534
		ACTACCACATTGCTTTATTGTAC	Reverse	23	34.8	51.0	
I	9	TATCACCCAGGAGCCAGG	Forward	18	61.1	56.3	520
		CCCACATGCCAGGACAAT	Reverse	18	55.6	55.4	

Note: Targets A, B, and C are not full-length exons; they are parts of exon 1 of *CYP2D6*, combining to give the full length of the exon. Targets D and F are stretches of DNA incorporating exons 2 and 3, and exons 5 and 6 of *CYP2D6*, respectively. In this study, these DNA segments are called "targets" (followed by the designated alphabet) for convenience.

For each of the targets listed in Table 2.1, DNA of 50 ng was amplified in a total volume of 25 µL, with 10µM of each primer (Integrated DNA Technologies, Iowa, USA) and 2X GoTaq Green Master Mix (Promega). Elution 1 DNA samples were used in this analysis.

The PCR tubes were placed into the wells of the BioRad T100 thermal cycler such that the targets would be amplified using the following cycling conditions: 5 minutes of initial denaturation at 95˚C, 30 cycles of denaturation at 95˚C for 30 seconds, 30 cycles of annealing

at 62.9˚C (for primers A, C, and I) or 55.3˚C (for the rest of the targets) for 30 seconds, 30 cycles of elongation at 72˚C for 30 seconds, and, finally, 5 minutes of final extension at 72˚C.

To determine if all the target segments were successfully amplified by PCR, agarose gel electrophoresis was performed. A 1.5% w/v agarose gel was prepared by dissolving agarose powder (Sigma-Aldrich, Missouri, USA) into Tris-Borate-EDTA (TBE) buffer, after which 0.01% v/v of SYBR® Safe nucleic acid gel stain (Thermo Fisher Scientific, Massachusetts, USA) was added to the resultant solution. The resultant gel was placed in a BioRad electrophoretic tank filled with TBE buffer and the PCR products were loaded (5 µL) into the wells. Electrophoresis was performed for 80 minutes at 100 Volts and 400 mAmp.

B) Determining the sequences of the amplified PCR products by Sanger sequencing

Sanger sequencing was performed on PCR products. This necessitated a preparatory post-PCR clean up step in which primers, deoxynucleotide triphosphates (dNTPs), and chaotropic salts were filtered out of the samples by following the instructions of the manufacturer – Nucleofast® 96 PCR (Macherey-Nagel, Düren, Germany). BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, California, USA) was used for sequencing. The manufacturer's protocol was followed to sequence the forward and reverse complements of targets A, B, C, E, and I; the reverse complements of targets D and H, and the forward complements of targets F and G. This procedure was performed by the University of Stellenbosch's Central Analytical Facility.

The resultant sequences were viewed using the ChromasLite version 2.4.4 software (Technelysium, South Brisbane, Australia). The BioEdit version 7.2.6 Sequencing Alignment Editor and Analysis Program (Hall, 1999) was used to compare the obtained sequences to the reference *CYP2D6* sequence, where multiple sequence alignment was performed using ClustalW with a bootstrap of 1000. The variants on the sequences were noted and their functional significance assessed by comparison to the variants documented on the Ensembl database (Release 90, August 2017).

2.5. Analysis of results

The data produced from the experiments described above was divided into three datasets based on the blood collection tubes that were used: purple-top, grey-top, and red-top tubes. The data was also divided according to the post-collection processing that the samples were subjected to, splitting the data into two categories: Tox samples and No-Tox samples. Each of the 30 cases was represented in terms of these groups of data, which formed the basis for the analysis that was carried out. The overall analysis was based on the comparison of these datasets and statistical tests were employed to compare data in some of the variables investigated.

The Stata® Data Analysis and Statistical Software (StataCorp, Canada, USA) was used for statistical analysis. The Shapiro Wilk test was performed to assess if the data was normally distributed. To test if there was a significant difference between the median values of the compared datasets, the Wilcoxon Signed Rank test was used. The tests were performed for DNA concentration, sample purity, and degradation index, where the level of significance (α) was set at 0.05. The details of the statistical analysis are provided in the statistical report in Appendix D.

CHAPTER 3: RESULTS

3.1. DNA quantification by qPCR

For all the blood specimens that were collected, DNA was extracted and quantified by qPCR. The aim of this analysis was to assess if the storage of blood specimens in the different collection tubes affected the quantity of the DNA in the blood, and whether handling samples in a toxicological environment has any adverse effect on endogenous DNA. The results of this analysis are presented in Figure 3.1. Of the three DNA targets (large autosomal, small autosomal, and Y chromosome) amplified by the Quantifiler™ Trio DNA Quantification system, the large autosomal target was chosen for this analysis, as its size (214 bp) is closest to the lengths of the sequences targeted in the intended toxicogenetic analysis.

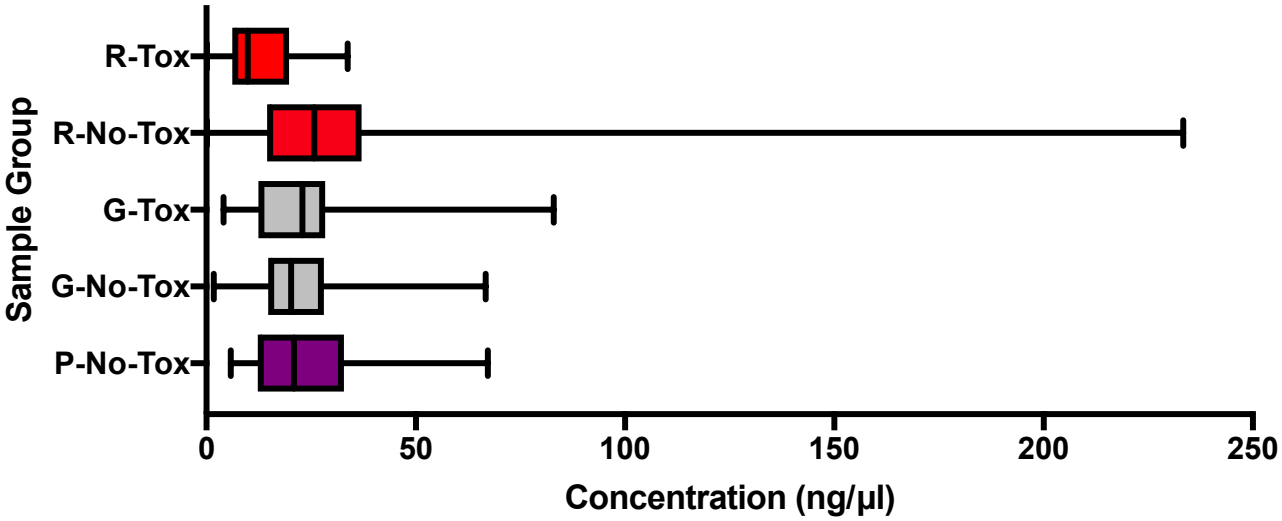


Figure 3.1: DNA Concentration assessed by qPCR. Postmortem blood samples (4 mL) were collected into three types of blood collection tubes: purple-top (P), grey-top (G), and red-top (R) tubes. The blood samples were stored at 4˚C and DNA was isolated within 72 hours of sample collection from the No-Tox samples. The G-Tox and R-Tox tubes were handled in a toxicological environment (at 4˚C) and DNA was extracted approximately 16 weeks after blood collection. The QiaAmp DNA Investigator's Kit (Qiagen, Hilden, Germany) was used for DNA extraction. The DNA samples were stored at 4˚C until the Quantifiler™ Trio DNA Quantification Kit (Applied Biosystems, California, USA) was used to quantify human DNA on a 7500 Real-Time PCR System (Applied Biosystems, California, USA).

The effect of the additives in the tubes (or lack thereof in the red-top tubes) was assessed by separating the tubes into "Tox" and "No-Tox" categories and performing an inter-tube type comparison within each category. The researchers noted an abnormally high DNA

concentration for 06R-No-Tox (233.4 ng/µL). However, because the comparison between the different groups of samples was based on median concentrations, the anomalous concentration did not affect the overall analysis of the data. For the No-Tox group, the red-top tubes produced DNA samples whose median concentration was significantly higher than those of the P-No-Tox ($p = 0.049$) and G-No-Tox ($p = 0.009$) samples. The R-No-Tox samples had DNA concentrations higher than all of the groups analysed in the study, with a median concentration of 25.8 ng/µL. Figure 3.1 also shows that the median concentrations of the purple-top (20.9 ng/µL) and the grey-top (20.3 ng/µL) tubes were not significantly different ($p = 0.530$).

However, when the Tox samples were compared, the grey-top tubes (median = 22.9 ng/µL) were shown to give DNA samples that were significantly higher ($p = 0.002$) in concentration than the red-top tubes (median = 9.9 ng/µL). The R-Tox samples produced the lowest DNA concentrations out of all the groups that were analysed by qPCR.

When intra-tube type analysis was performed, the R-No-Tox group was found to produce DNA concentrations that were significantly higher ($p < 0.001$) than those of the R-Tox group median. When the grey-top tubes were compared, the reverse of what was seen with the red-top tubes was observed: the G-Tox group produced a median concentration of 22.9 ng/µL, which was higher than the 20.3 ng/µL of the G-No-Tox group, but this difference was not significant ($p = 0.614$).

The gross concentration of nucleic acid content in all of the samples was also assessed by spectrophotometry, the results of which are presented in the supplementary data (Appendix C, Section C.1). Here, trends similar to those observed in qPCR quantification were seen in that the R-No-Tox tube produced the highest DNA concentration and the grey-top tubes as well as the purple-top tube produced DNA concentrations that were not significantly different (Figure S1). The major difference between the two datasets, however, is that the R-Tox tube produced the second highest median concentration of DNA when assessed by spectrophotometry, which contradicts the observation made when DNA was quantified by qPCR.

3.2. Assessment of the contamination of DNA by forensic DNA profiling

Forensic DNA profiling is normally performed for the purpose of identification, where allele peaks in one 'unknown' DNA profile are compared to allele peaks in another DNA profile of known origin. However, in this study, the DNA profiles were produced solely to see if a mixed profile would be observed in the electropherograms produced from DNA samples whose source blood had been handled in a toxicology laboratory – an environment whose major precautionary focus is not the contamination of samples with extraneous DNA.

No DNA profile was observed for the negative control sample (data not shown). This is taken as an assurance that if DNA contamination were to be observed for any of the study cases in the form of a profile mixture, it would not have been introduced in the process of conducting the analysis. No DNA profile mixture was observed in any of the electropherograms examined for this analysis. Figure 3.2 is portion of a DNA profile for one of the study cases and is presented as an example of the lack of contamination/DNA profile mixture asserted above. A full DNA profile is not shown here to protect the identity of the participant.

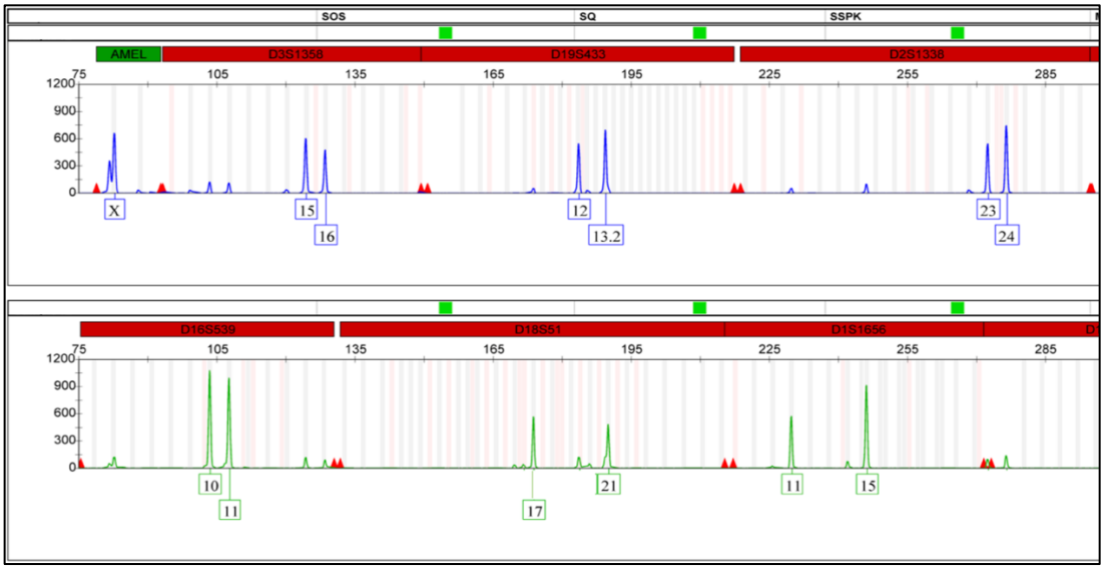


Figure 3.2: A portion of a DNA profile for one of the study cases. Postmortem blood samples (4 mL) were collected into two types of blood collection tubes: grey-top and red-top tubes. The blood samples were handled in a toxicological environment (at 4˚C) and DNA was extracted approximately 16 weeks after blood collection using the QiaAmp DNA Investigator's Kit (Qiagen, Hilden, Germany). The PowerPlex® ESI 16 System (Promega, Wisconsin, USA) was used to amplify 16 STR markers on the isolated DNA, 10 of which are shown in the figure. The samples were run at 60 ˚C on a Genetic Analyser 3130 *xl* (Applied Biosystems, California, USA) with the following running conditions: injection time – 5 seconds, injection voltage – 3 kV, and run time – 1 500 seconds.

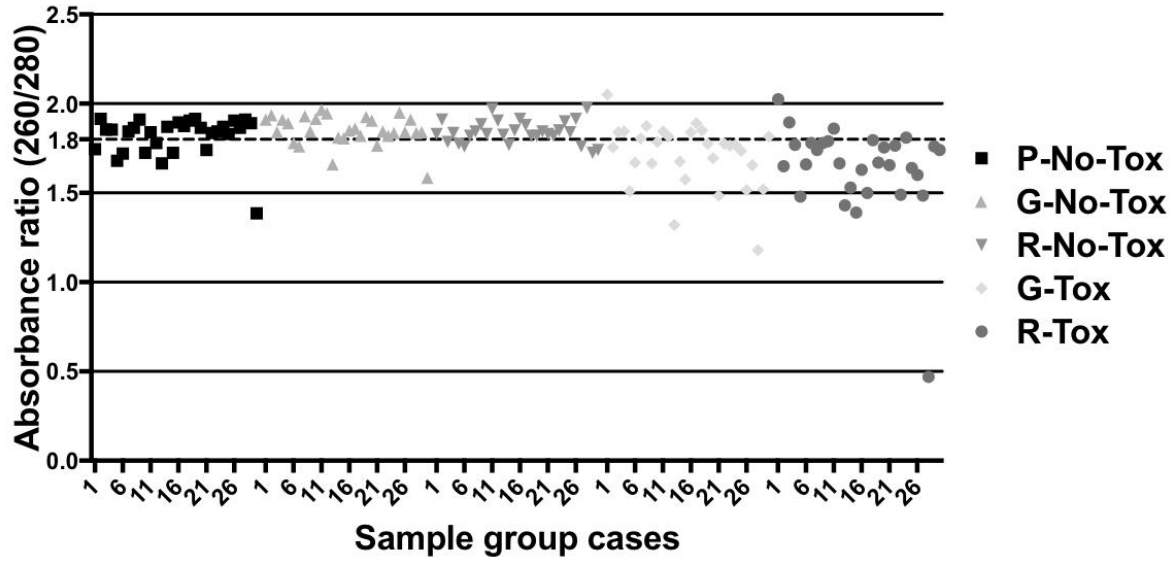
It is worth noting that instrumental anomalies (pull-ups and dye blobs) were observed in many of the electropherograms, however, all of these artefactual peaks were accounted for and their sources identified. The handling of samples in a toxicological environment, therefore, did not cause contamination of the study samples with extraneous human DNA.

3.3. Assessment of DNA purity by spectrophotometry

Spectrophotometry was also used for quality assessment, wherein the absorbance ratios 260/280 and 260/230, indicating the contamination of isolated DNA samples by peptide molecules and chaotropic salts, respectively, were recorded to determine the effect of the different additives in the different collection tubes on the purity of the isolated DNA (purple-top versus grey-top versus red-top tubes). The analysis also examined if the handling of samples in a toxicology laboratory reduced the purity of the isolated DNA (Tox versus No-Tox samples).

For both 260/280 and 260/230, a DNA sample was considered to be pure only if the absorbance ratio was above 1.8 (denoted by a dashed line in Figure 3.3.A. and Figure 3.4.A.) (Desjardins and Conklin, 2010). Therefore, for this analysis, each sample was examined to see if its absorbance ratio passes this threshold, above which purity was declared. For 260/280 (Figure 3.3) and 260/230 (Figure 3.4) absorbance ratios, the scatter of the 30 samples in each group is represented in the chart shown in A, while the bar graph in B shows the proportion (n/30) of samples whose absorbance ratios were equal to or above 1.8.

A



B

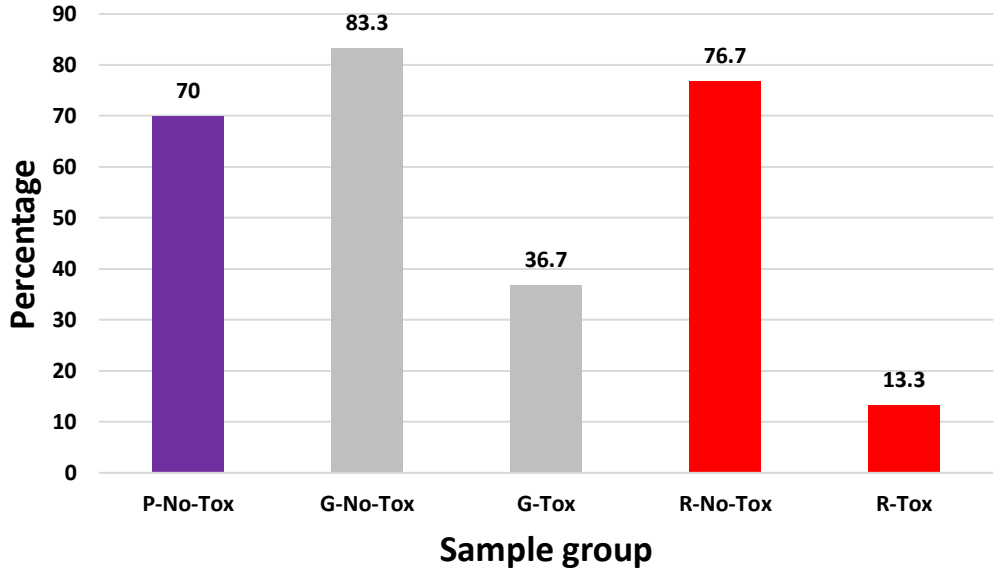


Figure 3.3: Absorbance ratio 260/280 assessed by spectrophotometry. **A**: the scatter of the 30 samples in each sample group; **B**: the percentage (n/30) of samples whose absorbance ratios were above the value 1.8 (denoted by a dashed line in **A**). Postmortem blood samples (4 mL) were collected into three types of blood collection tubes: purple-top (P), grey-top (G), and red-top (R) tubes. The blood samples were stored at 4˚C and DNA was isolated within 72 hours of collection from the No-Tox samples. The G-Tox and R-Tox tubes were handled in a toxicological environment (at 4˚C) and DNA was extracted approximately 16 weeks after blood collection. DNA was extracted using the QiaAmp DNA Investigator's Kit (Qiagen, Hilden, Germany) and NanoDrop 2000 (Thermo Scientific, Massachusetts, USA) was used to assess the 260/280 absorbance ratio.

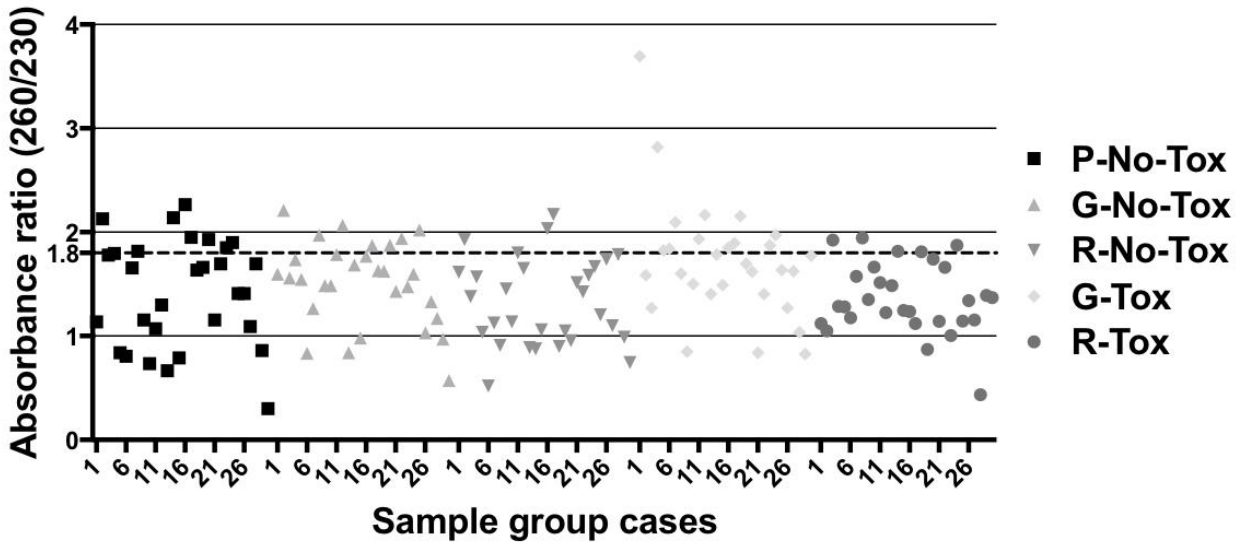
Figure 3.3.A shows that, regarding protein contamination, the samples from all three tube types were similar in purity when they were not subjected to a toxicological environment.

Statistically, there was not enough evidence to reject the null hypothesis; the p-values for the comparisons of the median absorbance ratios between (i) P-No-Tox and G-No-Tox, (ii) P-No-Tox and R-No-Tox, and (iii) G-No-Tox and R-No-Tox were 0.81, 0.334, and 0.484, respectively. An overall examination of these (No-Tox) samples shows that, generally, there were more samples that were relatively free from protein contamination than those that were not (Figure 3.3.B). In the group of samples with the least number of pure samples (P-No-Tox), 70% of the samples were pure, with the other groups, R-No-Tox and G-No-Tox, having higher proportions of 76.7% and 83.3%, respectively.

In contrast, Tox samples had relatively lower purity. This is illustrated in Figure 3.3.A by the data points moving away from the gridline of 2 in the Y-axis, and closer to the gridline marking 1.5. This is further confirmed by the purer group (G-Tox) only having 36.7% of its samples passing the mark of 1.8, while this proportion was 13.3% for the R-Tox group (Figure 3.3.B).

Regarding contamination with chaotropic salts (260/230), all the samples had relatively lower purity (Figure 3.4). In contrast to what was seen in 260/280, when the G-No-Tox and R-No-Tox samples were compared to the Tox samples regarding contamination with chaotropic salts, the samples that were handled in a toxicological environment appeared to have higher purity. The No-Tox samples were dominated by the P-No-Tox group, with 26.7% of its samples being pure, while the G-No-Tox and the R-No-Tox groups had 23.3% and 13.3% of their samples, respectively, being pure. Of the Tox groups, the grey-top tubes produced the higher proportion of pure DNA samples: 40%. This was also the highest proportion recorded for the whole analysis, which is an indication that, generally, there were more samples that were impure than those that reached the 1.8 mark.

A



B

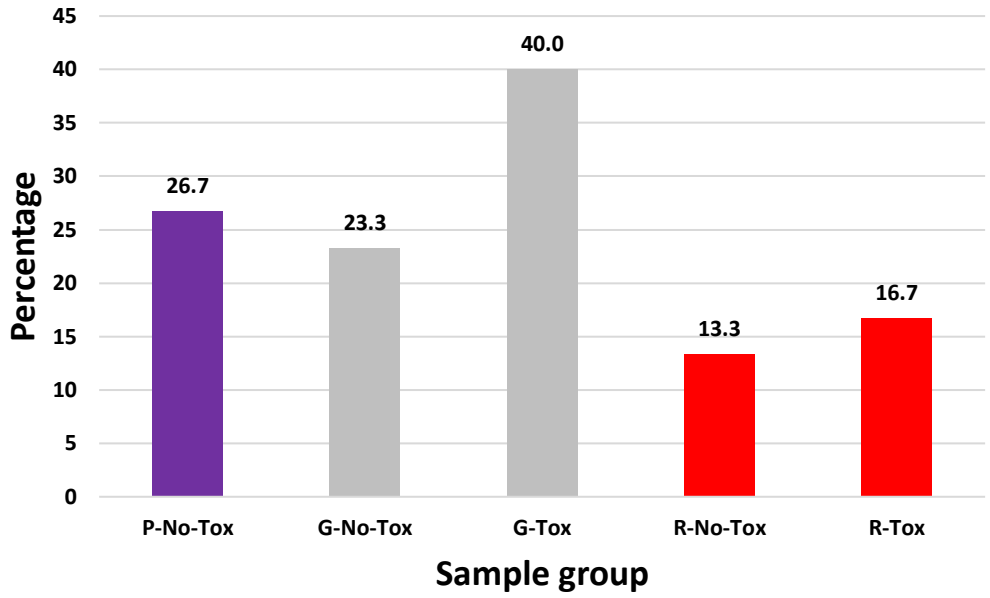


Figure 3.4: Absorbance ratio 260/230 assessed by spectrophotometry. **A**: The scatter of the 30 samples in each sample group; **B**: The percentage (n/30) of samples whose absorbance ratios were above the value 1.8 (denoted by a dashed line in **A**). Postmortem blood samples (4 mL) were collected into three types of blood collection tubes: purple-top (P), grey-top (G), and red-top (R) tubes. The blood samples were stored at 4˚C and DNA was isolated within 72 hours of collection from the No-Tox samples. The G-Tox and R-Tox tubes were handled in a toxicological environment (at 4˚C) and DNA was extracted approximately 16 weeks after blood collection. DNA was extracted using the QiaAmp DNA Investigator's Kit (Qiagen, Hilden, Germany) and NanoDrop 2000 (Thermo Scientific, Massachusetts, USA) was used to assess the 260/230 absorbance ratio.

3.4. Assessment of the degradation of DNA by qPCR

The extracted DNA was quantified using qPCR to determine the degradation index, as a means to assess the quality/integrity of DNA in each sample collected. Since degradation index in this context is defined as the concentration of the small autosomal target divided by the concentration of the large autosomal target, the principle of this analysis is such that if the degradation index is equal to or less than 1, it implies that the small autosomal target was not amplified preferentially, which is indicative of DNA which is not degraded. Presented in Figure 3.5 are the median degradation indices and 95% confidence intervals for all the sample groups analysed.

It was noted that all the degradation indices were below 1 (denoted by a dashed line in Figure 3.5), suggesting an absence of degradation in all of the groups analysed. However, a large variability between the groups was seen: the median degradation indices in the red-top tube samples (0.80 for R-No-Tox and 0.68 for R-Tox) were significantly lower than those of the grey-top tube samples (0.87 for G-No-Tox and 0.84 for G-Tox) for both the No-Tox ($p = 0.009$) and the Tox ($p < 0.001$) groups. The median degradation index of the purple-top tubes (0.85) was not significantly different to that of the G-No-Tox group ($p = 0.797$) but significantly higher than that of the R-No-Tox group ($p = 0.043$).

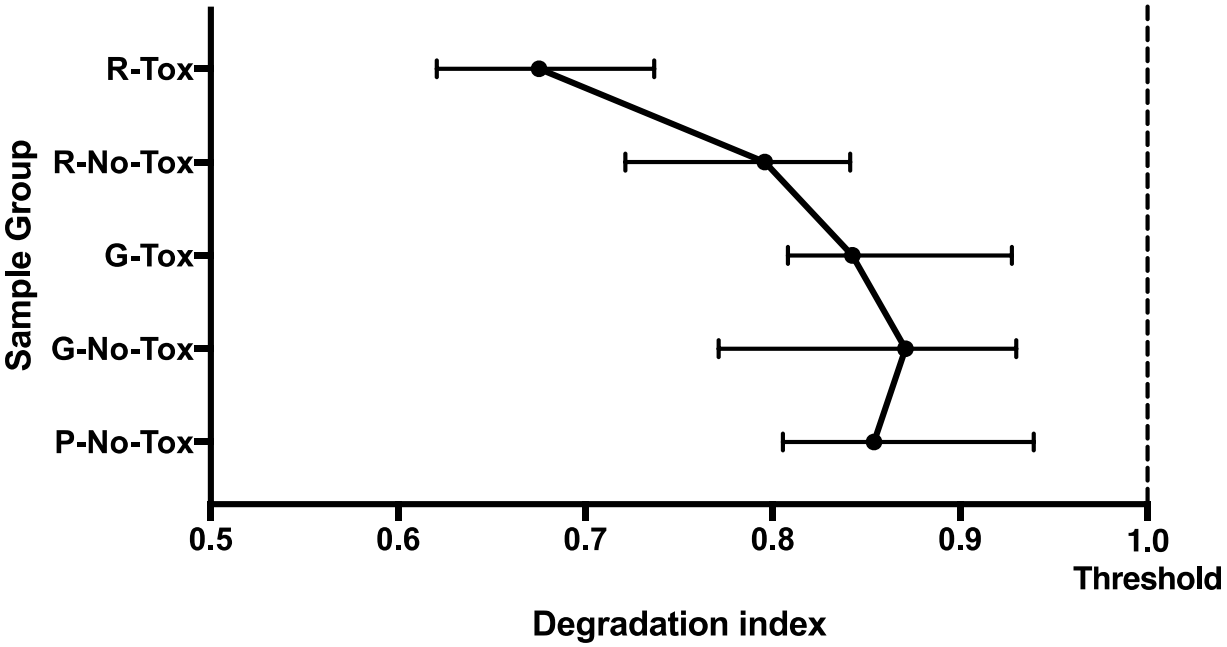


Figure 3.5: Degradation indices of isolated human DNA. Postmortem blood samples (4 mL) were collected into three types of blood collection tubes: purple-top (P), grey-top (G), and red-top (R) tubes. The blood samples were stored at 4˚C and DNA was isolated within 72 hours of collection from the No-Tox samples. The G-Tox and R-Tox tubes were handled in a toxicological environment (at 4˚C) and DNA was extracted approximately 16 weeks after blood collection. The QiaAmp DNA Investigator's Kit (Qiagen, Hilden, Germany) was used for DNA extraction. The DNA samples were stored at 4˚C until the Quantifiler™ Trio DNA Quantification Kit (Applied Biosystems, California, USA) was used to determine the degradation indices of the endogenous DNA on a 7500 Real-Time PCR System (Applied Biosystems, California, USA).

Intra-tube type comparison of degradation indices was also performed to assess the effect that the handling of blood samples in a toxicological environment has on DNA. There was no significant difference ($p = 0.829$) in the median degradation index between the grey-top tube samples that were handled in a toxicology laboratory (0.84) and those that were not (0.87). In contrast, the median degradation index of the R-Tox samples (0.68) (which was the lowest for all the groups that were examined in this analysis) was significantly lower ($p < 0.001$) than that of the R-No-Tox samples (0.80). Despite this observed intra-tube difference between R-Tox and R-No-Tox, it was noted that the red-top tubes produced the lowest degradation indices for the whole analysis, regardless of whether or not the samples were handled in a toxicological environment (Figure 3.5).

3.5. Assessment of the quality of DNA by Sanger sequencing

For this experiment, DNA sequences were presented in the form of electropherograms, and the comparisons were made on the basis of peak shape, background noise, and the ability of the software to accurately call the base represented by the peaks (which is influenced by peak shape and background noise). However, agarose gel electrophoresis was first performed to assess whether the *CYP2D6* targets were successfully amplified and whether there was any difference in the produced amplicons between the G-Tox (GT) and the R-Tox (RT) samples (Figure 3.6).

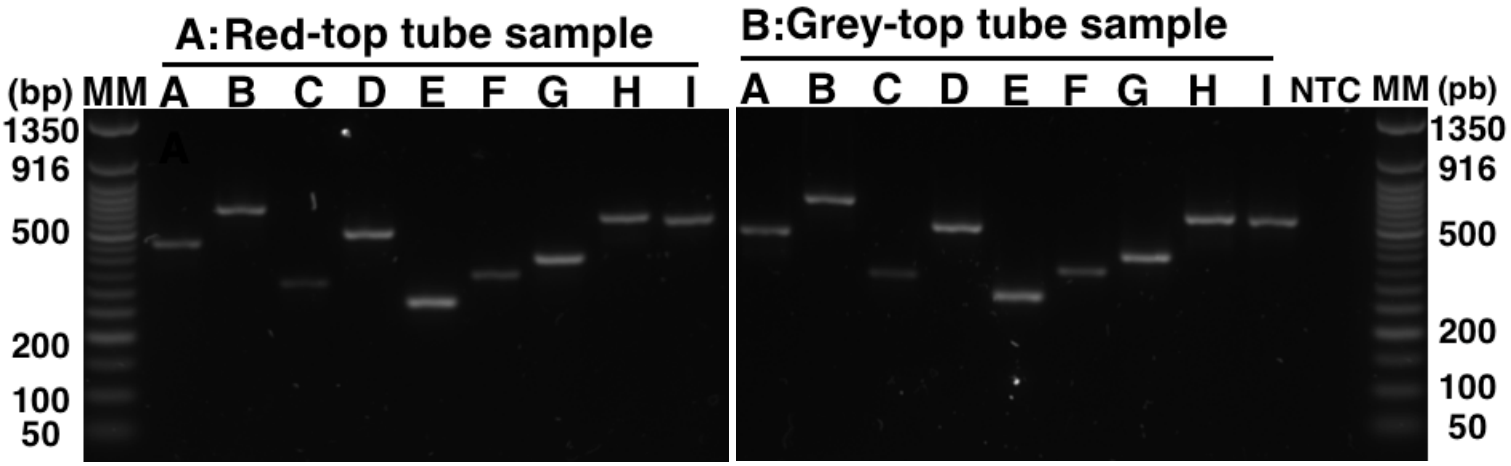


Figure 3.6: PCR amplification of all the *CYP2D6* targets for the GT and RT samples. Postmortem blood samples (4 mL) were collected into two types of blood collection tubes: grey-top (GT) and red-top (RT) tubes. The blood samples were handled in a toxicological environment (at 4˚C) and DNA was isolated approximately 16 weeks after collection using the QiaAmp DNA Investigator's Kit (Qiagen, Hilden, Germany). Aliquots of the extracted DNA were amplified by PCR on a BioRad T100 thermal cycler (California, USA) using primers designed in-house and a GoTaq® Green Master Mix (Promega, Wisconsin, USA). The amplicons were resolved in a 1.5% w/v agarose gel, where, for gel A, the wells were loaded as follows: well 1(MM): Quick-Load® 50 bp Ladder (New England Biolabs, Massachusetts, USA), and well 2–10: targets A–I for the R-Tox sample. For gel B, the wells were loaded as follows: well 1–9: targets A–I for the G-Tox sample, well 10 (NTC): no template control sample, and well 11 (MM): Quick-Load® 50 bp Ladder. Electrophoresis was performed for 80 minutes at 100 V and 400 mAmp.

As can be seen in lane "NTC" of gel B in Figure 3.6 – where the no template control sample was loaded – there were no bands produced for the sample that served as a negative control, suggesting that the procedure that the samples were taken through did not introduce contamination in the form of human DNA. All the 9 targets (A–I) were successfully amplified for both the G-Tox and the R-Tox sample (Figure 3.6). Based on the intensities of the bands

produced, for every target, there was no remarkable difference in the amount of DNA that was amplified between the G-Tox sample and the R-Tox sample.

For the 9 targets that were examined by Sanger sequencing, the DNA sequence produced with DNA from the G-Tox sample was compared to the DNA sequence produced with DNA from the R-Tox sample (Figure 3.7). Represented with the electropherograms shown in Figure 3.7 are the (I) forward and (II) reverse complements of target A. The top panel shows the electropherogram that was obtained when sequencing was performed using DNA from the G-Tox (GT) sample and the bottom panel shows the electropherogram that was obtained when sequencing was performed using DNA from the R-Tox (RT) sample.

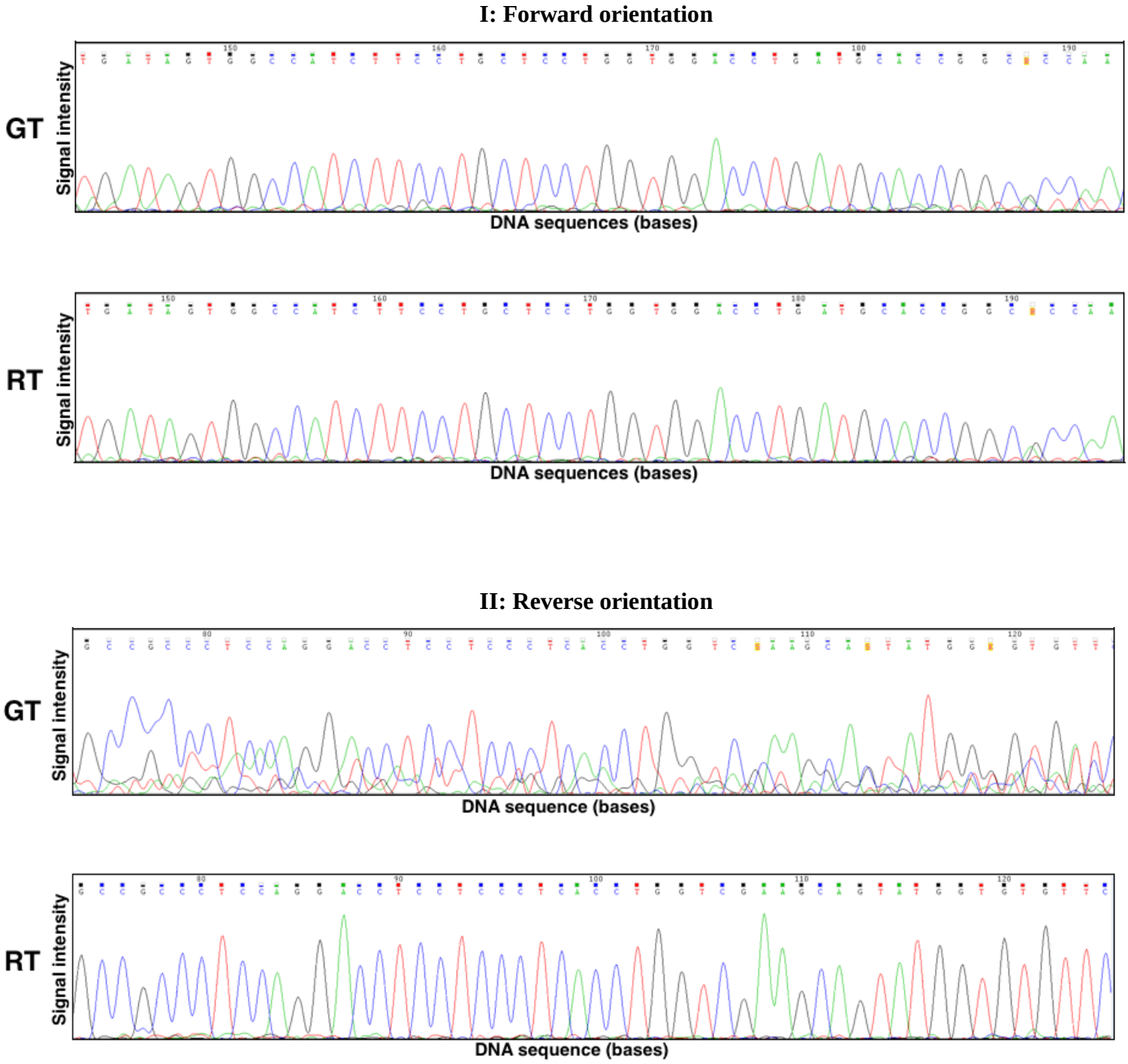


Figure 3.7: A portion of the DNA sequence of target A. Postmortem blood samples (4 mL) were collected into two types of blood collection tubes: grey-top (GT) and red-top (RT) tubes. The blood samples were handled in a toxicological environment (at 4˚C) and DNA was isolated approximately 16 weeks after collection using the QiaAmp DNA Investigator's Kit (Qiagen, Hilden, Germany). The targets of *CYP2D6* were amplified by PCR using primers designed in-house. The sequences of the (I) forward complement and the (II) reverse complement of target A were determined by a Sanger sequencing method that utilised the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA).

Looking at the background noise in Figure 3.7.I, it can be seen that the R-Tox sample produced an electropherogram of similar quality compared to the one produced by the G-Tox sample. In contrast, Figure 3.7.II shows two electropherograms in which the DNA sequence produced by the R-Tox sample is substantially better in quality than that produced by the G-Tox sample. The difference between the two electropherograms is so drastic that numerous inconsistencies in terms of the bases called by the software were observed. This is especially demonstrated by the three artefactual SNPs produced in the GT electropherogram as a result of its poor quality, which would complicate the process of analysing the DNA sequence. When such situations were encountered with other comparisons (Appendix C, Section C.2: Figure S2–S9), it was under this premise that the quality of one DNA sequence was declared to be better than the other. Figure 3.8 shows the results of the comparisons made for all the targets (A–I) examined in the study.

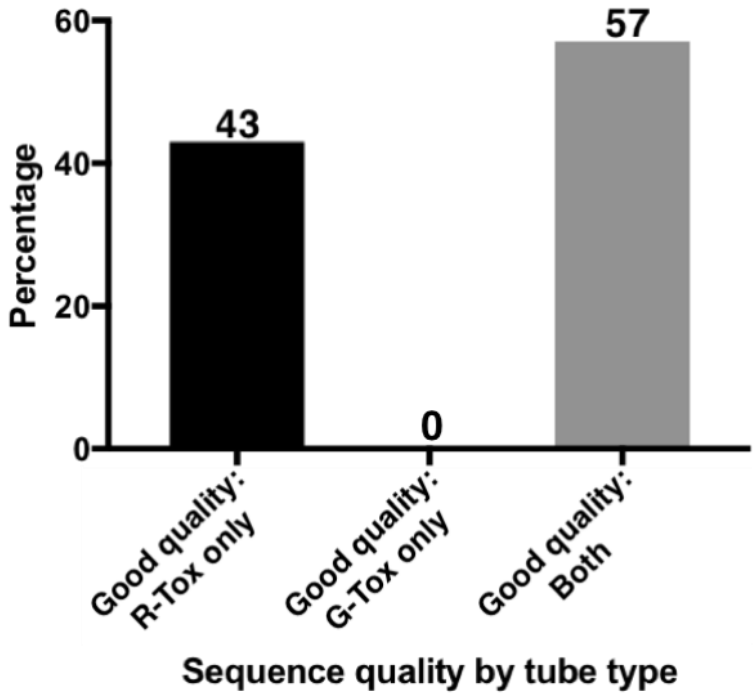


Figure 3.8: **Comparison of the quality of DNA sequences between GT and RT**. Postmortem blood samples (4 mL) were collected into two types of blood collection tubes: grey-top (GT) and red-top (RT) tubes. The blood samples were handled in a toxicological environment (at 4˚C) and DNA was isolated approximately 16 weeks after collection using the QiaAmp DNA Investigator's Kit (Qiagen, Hilden, Germany). The targets of *CYP2D6* were amplified by PCR using primers designed in-house. The sequences of the forward complement and the reverse complement of target A were determined by a Sanger sequencing method that utilises the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA).

As shown in Figure 3.8, the sequences from both tubes were of good quality in 57% of the electropherograms produced. The sequence was good quality for the R-Tox sample and poor quality for the GT sample in 43% of the electropherograms generated. In none of the electropherograms was the DNA sequence produced from the G-Tox sample of better quality than that produced from the R-Tox sample.

3.6. Application of the toxicogenetic analysis

The analysis on the basis of Sanger sequencing had shown the red-top tube to produce DNA samples better suited for the generation of sequence electropherograms. To test the applicability of this result, toxicogenetic analysis was performed using the 22R-Tox sample, where the assay designed in-house was applied to amplify the exons of *CYP2D6*, after which they were analysed by Sanger sequencing. The variants in the resultant sequences were noted, and those for which the corresponding haplotypes were determined are presented in Table 3.1.

Table 3.1: Variants detected for case 22 and the corresponding properties.

RefSNP (rs) Number	Corresponding Haplotype	Observed Genotype	Nucleotide Change M33388.1 ATG=1	Enzyme Activity
rs28371718	*CYP2D6*1D*	C/A	2575C>A	Normal
rs774671100	*CYP2D6*13*	G/A	137_138insT	None
rs769157652	*CYP2D6*27*	G/A	3853G>A	Normal
rs28371696	*CYP2D6*43*	G/A	77G>A	Normal
rs77913725	*CYP2D6*86*	G/A	2606G>A	Unknown
rs1135828		T/A	2610T>A	

A total of 29 SNPs was detected in the *CYP2D6* gene of the chosen case. Of the 29 variants, only 6 translated into recognised haplotypes (Table 3.1). Most of the detected haplotypes (*CYP2D6*1D*, *CYP2D6*27*, and *CYP2D6*43*) do not cause a change in the "normal" activity of the CYP2D6 enzyme. In contrast, the presence of the *CYP2D6*13* haplotype completely eradicates the activity of the allele on which the rs769157652 SNP is located. The rs769157652 and rs1135828 combined to give the *CYP2D6*86* haplotype, whose effect on the activity of the enzyme is not known.

3.7. Correlation analyses: assessment of the effect of period differences

It should be noted that the nature of the current study is such that participants were recruited on different days, with the period between death and the collection of blood samples ranging from 0 to 7 days. Also, the extraction of DNA within 72 hours of blood collection for the No-Tox samples made the period between death and DNA extraction relatively short when compared to the Tox samples, wherein the period between death and the extraction of DNA ranged from 7 to 99 days. To account for this difference, correlation tests were performed for the Tox samples to assess whether the trends observed in the results presented in this chapter could be ascribed to the described period differences (Appendix C). In the two sets of data that were generated, no correlation between time and concentration, degradation index, or absorbance ratio was found for both the period between death and the collection of blood samples (Appendix C, Section C.3), and the period between death and the extraction of DNA (Appendix C, Section C.4).

An overview of all the analyses that were performed in the study and the results that were obtained is provided in Table 3.2.

Table 3.2: Overview of the analyses performed and the results obtained in the study.

Analysis/Experiment	Goal	Result/Highlight of the analysis
DNA quantification by qPCR.	To test the effect of the different additives in the different blood collection tubes on the concentration of DNA.	DNA concentration decreased significantly in blood stored in red-top tubes and handled in a toxicological environment while it remained relatively constant when stored in a grey-top tube.
DNA quantification by Spectrophotometry.		Overall, red-top tubes produced the highest DNA concentrations, while DNA concentration remained constant in the samples stored in grey-top tubes.
Assessment of the contamination of DNA by forensic DNA profiling.	To test whether handling blood collection tubes in a toxicological environment exposes the blood to contamination with human DNA.	No DNA profile mixture was observed in any of the electropherograms examined for this analysis, indicating absence of contamination with human DNA.
Assessment of DNA purity by spectrophotometry.	To test whether any (and the extent to which) the different additives in the different blood collection tubes predisposes the collected DNA to the introduction of unwanted impurities.	In both grey- and red-top tubes, purity (in terms of contamination with peptide molecules) decreased when the samples were handled in a toxicological environment while it decreased in terms of contamination with chaotropic salts under the same conditions.
Assessment of the degradation of DNA by qPCR.	To test whether the storage of blood in the different blood collection tubes causes DNA degradation.	All the tube types produced degradation indices below 1. Degradation index tended to decrease when samples were handled in a toxicological environment.
Assessment of the quality of DNA by Sanger sequencing.	To test which of the blood collection tubes normally used for the storage toxicological samples is best suited to the storage of blood from which Sanger sequence is subsequently to be performed.	The DNA derived from the red-top tubes produced only DNA sequences of good discernible quality, while the DNA derived from the red-top tubes produced DNA sequences of good discernible quality in only 57% of the instances.
Application of the toxicogenetic analysis.	To test whether samples stored in the tube type that performed better in the analysis above can be used for the intended toxicogenetic analysis.	The sequences produced from the DNA derived from red-top tubes were successfully used to determine the haplotyes necessary to ascertain the CYP2D6 metabolic phenotype of the individual in question.
Correlation analyses: assessment of the effect of period differences.	To test if a correlation exists between the results observed and the period differences between death and blood collection, and between death and DNA extraction.	No correlation between time and concentration, degradation index, or absorbance ratio was found for both the period between death and the collection of blood samples, and the period between death and the extraction of DNA.

CHAPTER 4: DISCUSSION AND CONCLUSION

Given that the prevalence of drug use in South Africa is one of the highest in Africa (Otu, 2011), an examination that elucidates the extent to which certain genes contribute to drug-related deaths may prove significant in a local forensic context. Case reports on oxycodone (Jannetto *et al.*, 2002), tramadol (Levo *et al.*, 2003), citalopram (Holmgren *et al.*, 2004), and amitriptyline (Koski *et al.*, 2006) toxicity have shown the significance of postmortem toxicogenetic analysis. The literature reviewed indicated that there is value of a toxicogenetic assay to demonstrate the inadvertent contribution of an individual's genetic make-up in the production of ADRs. The debate about the inclusion of this test as a standard component of postmortem investigation is centered on whether or not it is necessary, feasible and applicable as a routine test (Koski *et al.*, 2006).

No information has been reported on the pre-analytical process the examination involves. This is crucial, as a successful integration of toxicogenetic analysis would include a tested, validated and standardised approach of the pre-analytical process for both the toxicological and genetic components of the examination. The careful consideration of this aspect of the toxicogenetic analysis is informed by the knowledge that the majority of laboratory errors have little to do with validated instrumental analyses, but rather derive from issues in the pre-analytical process (Skopp, 2004).

This study aimed to address this by looking at one of the most important aspects of the pre-analytical process: whether the collection and post-collection processing of the samples handled in a toxicological environment compromise the quality of the DNA in such a way that it cannot be used for the intended subsequent genetic analysis. Genetic analyses were utilised for this purpose, the results of which are discussed below.

4.1. DNA quantification

The quantification of extracted DNA by qPCR showed that the red-top tubes that had not undergone toxicological analysis produced the highest DNA yield out of all the groups that were compared. This was even higher than the control group – the purple-top tube samples. The observation may be attributed to the additives in the different tube types, as it is the only

distinguishing factor between them. The mechanism of how this takes place was not investigated in the current study and can only be hypothesised from what was observed and what is known about the tubes used in the study. A possible explanation for the variation in DNA concentration in the different tube types is that the additives in the purple-top tubes and the grey-top tubes are involved in a process that results in the structural damage/conformational change of DNA such that it cannot be amplified optimally. In the case of the grey-top tubes, this supposition is supported by studies that have postulated that the fluoride ion denatures DNA at the level of the secondary structure (Liu *et al.*, 2017).

Interestingly, the red-top tube produced the lowest DNA concentration when subjected to toxicological conditions, whereas there was no significant change in concentration in the grey-top tubes when they were handled in a toxicological environment. This, again, can be attributed to the red-top tube's lack of additives. Authors such as Nazir *et al.* (2011) and Johnson and Ferris (2002) have argued that the *nucleases* that are released during the process of cell lysis in postmortem blood result in DNA fragmentation (Johnson and Ferris, 2002; Nazir *et al.*, 2011). The EDTA contained in purple-top tubes prevents DNA fragmentation through the chelation of Mg^{2+} (Lahiri and Schnabel, 1993), which is required by some *nucleases* as a cofactor. In the grey-top tube, potassium oxalate acts as an anti-coagulant, while the activity of sodium fluoride is centred on its ability to inhibit the activity of microorganisms such as *Candida albicans* and *Escherichia coli* (Yajima *et al.*, 2006).

The red-top tube is a plain vacutainer and, therefore, has no preventative measure against biocatalysis and/or microbial action, both of which may contribute to the degradation of DNA and the observed reduction in DNA concentration upon handling in a toxicological environment (Nakao and Ogata, 1963). The argument of uninhibited microbial activity in the red-top tubes was supported by an observation made when DNA was quantified using spectrophotometry (Appendix C, Section C.1.). In the said analysis, the red-top tubes before and after toxicological analysis produced the two highest median DNA concentrations for the whole analysis (Figure S1). Since quantification with qPCR (which targets only human DNA) showed that DNA in the red-top tubes reduces significantly when handled in a toxicological environment, the observation made when spectrophotometry was applied suggests that the high DNA content that was detected in the red-top tube after toxicological analysis was not 100% of human origin. We hypothesise that the detected DNA is an aggregate of the human DNA

originally present in the blood and that of the microorganisms whose growth is uninhibited in the specimen. This is in line with the fact that spectrophotometry does not have a mechanism for distinguishing DNA from different species; it quantifies all the DNA present in a sample.

The similarity of concentrations between the grey-top tubes that were handled in a toxicological environment and those that were not can also be linked to microbial activity in the blood contained in the tubes. We postulate that the limited microbial activity in the grey-top tubes accounts for the observed consistency in DNA yield in the sense that there is restricted microbial growth affecting the human DNA in the blood. This was also confirmed in the spectrophotometric DNA quantification wherein the DNA obtained on samples that had undergone toxicological analysis was similar in concentration to that obtained for samples that had not been handled in a toxicology laboratory. Further research is required to test if the unhindered microbial activity has deleterious effects on postmortem blood and whether such an effect would substantially be mitigated by the inhibition exerted by sodium fluoride on certain species of microorganisms.

It must be noted, however, that regardless of the variation in concentration seen between different tube types and handling conditions, the concentrations obtained were sufficient to perform PCR, which is an essential step in the procedure of toxicogenetic analysis.

4.2. Assessment of the contamination of DNA by forensic DNA profiling

Blood specimens sampled for toxicological analysis are handled in a chemistry-based environment, which is not specifically designed for preserving the integrity of DNA. As such, to test whether the blood samples that underwent toxicological analysis had been contaminated with the DNA of another individual, forensic DNA profiling was performed on the Tox samples. It was expected that if any of the samples had been contaminated with DNA from another human, this would be indicated by the presence of more than two allele peaks in more than one locus of the generated DNA profile.

The study found that the handling of blood samples in a toxicological environment did not result in the contamination of the blood with extraneous DNA. The absence of DNA contamination in the blood samples can be ascribed to the fact that, although a toxicology

laboratory is not particularly concerned about the contamination of samples with DNA, laboratories working with biological specimens generally take precaution measures against human skin making contact with human blood specimens, as it is a health hazard. Toxicology laboratories are not an exception to this. In addition, biological specimens are usually sampled within biosafety cabinets to reduce risks to the analyst. This is an important finding because the DNA used in a toxicogenetic analysis must be free from DNA contamination, as the analysis is centered on analysing the sequence of the decedent's DNA to determine the corresponding metabolic phenotype.

We, therefore, recommend the performance of DNA profiling as a quality assurance step going forward. That is, DNA profiling should be performed prior to use of handled samples to distinguish any contamination with extraneous DNA before more expensive PCR and sequencing tests are performed. This is suggested as toxicology specimens may be sampled multiple times for both screening and quantitative analyses. This was only performed twice in this study. Therefore, while the samples in this study were not contaminated, samples in the future might be handled by different people in slightly different conditions, so we recommend this step for future samples so that if a mutation is found, then it can be attributed to the decedent and not a lab personnel.

4.3. Assessment of purity by spectrophotometry

When the purity of blood samples was assessed, the analyses of proteins and chaotropic salts presented results with opposing trends: for the assessment of contamination with proteins, samples handled in the toxicological environment decreased in purity; however, an increase in purity was observed in Tox samples when an examination for contamination with chaotropic salts was performed. Because absorbance ratio 280/260 is a function of DNA concentration, the reported significant decrease in DNA concentration for the red-top tube samples can be used to explain the observed decrease in purity.

In all of the groups examined, however, the purity in the red-top tubes was constantly lower than that observed for the grey-top tubes. The lack of additives in the red-top tubes cannot be used as an explanation for the observed lower purity, as purple-top tubes (which have EDTA as an additive) had purity lower than that observed in red-top tubes (regarding contamination

with peptide molecules). While the investigation into the cause of the variation observed in purity between different tube types was beyond the scope of this study, the potential effects of impurities in DNA samples could not be ignored.

The manner and extent to which high content of peptide molecules affects the process of toxicogenetic analysis is not known. Salts, ionic detergents, and phenols are known PCR inhibitors (Bessetti, 2007), some of which are reflected in the 260/230 absorbance ratio (Desjardins and Conklin, 2010). This is important to note because the procedure of the proposed toxicogenetic analysis involves a PCR step. Samples that have a high content of one or more of the compound types cited above, therefore, would not be favourable for the said genetic assay. However, such a premise implies that DNA samples from the red-top tubes – which were significantly lower in purity when compared to the grey-top tubes – would be amplified less efficiently in the PCR step. Interestingly, however, there was no apparent difference in the bands (in an agarose gel) representing amplicons produced by PCR from the grey-top and red-top tube samples (Figure 3.6).

4.4. Assessment of the degradation of DNA by qPCR

The results of the analysis that examined degradation index in this study suggested that all the sample groups produced DNA that was not degraded over the study period. The variation that was observed in degradation index existed within the range of non-degraded DNA. However, this variation was significant between the red-top tubes (Tox and No-Tox samples) and between the red-top tube group and the other tube types. The variation, therefore, cannot be neglected. While a degradation index above 1 suggests that the assay used for amplification was better able to amplify the smaller DNA target, indicative of DNA fragmentation, a degradation index substantially below 1 suggests that the larger DNA target was amplified preferentially. This cannot be read to signify DNA fragmentation.

Although no significant difference existed between the degradation indices recorded for the grey-top tubes before and after toxicological analysis, a decrease in this value was still noted in the Tox sample group, as was the case for the red-top tubes. This increase in the preferential amplification of the larger fragment when samples were handled in a toxicological environment is, therefore, not limited to a specific tube type. The only other experiment performed in the

study in which this trend – a similar change (increase or decrease) between the Tox and No-Tox samples for both the grey-top and the red-top tubes – was noted, was in the assessment of the purity of the DNA. The reason for the preferential amplification of the large autosomal target was not investigated in the current study, and it is not known whether it has any association with DNA purity. However, the cause could be hypothesised to be due to different GC contents for the large autosomal target and the small autosomal target, and the amplification conditions being slightly favourable to the amplification of the large target as a result of that.

What is denoted by the significant difference in the degradation index between the red-top tubes and the other tube types is that the presence of additives in the purple-top and grey-top tubes reduces the degree to which this phenomenon (of preferential amplification of the large autosomal target) occurs. Of utmost importance for the researchers, however, is whether or not and/or the extent to which this phenomenon negatively affects the ability to use the stored DNA for the intended toxicogenetic analysis – a question that was answered by the results of the analysis discussed below.

4.5. Assessment of the quality of DNA by Sanger sequencing

The toxicogenetic analysis for *CYP2D6* consists of three components: (1) amplifying the investigated DNA using the designed primers, (2) determining the sequence of the amplified segments by Sanger sequencing, and (3) analysing the sequences to determine the metabolic phenotype encoded by the gene. The aim of this analysis was to test whether it was possible to perform these three procedures on DNA derived from Tox samples. This is because it is envisaged that the designed toxicogenetic analysis would be performed following toxicological analysis, and most likely on samples retrieved from a toxicology laboratory.

For more than half of the electropherograms produced for this analysis, no substantial difference in sequence quality was observed between the G-Tox and R-Tox samples (Appendix B, Figure S1 – S8). All of these electropherograms were intelligible. However, the results also showed that in close to a half of electropherograms produced, the sequences were better in the samples from the red-top tube. Overall, the quality was poor in close to a half of the electropherograms produced by grey-top samples, while all of the electropherograms produced by the red-top tube samples were of good quality, enabling the interpretation of the sequences.

This provides an answer to the study question, as it shows that blood samples collected and stored in red-top tubes are suitable for use in a genetic analysis, following handling involved in the process of toxicological investigation over a period of 16 weeks. However, given the observed decline in DNA quantity in the red-top tubes, and the contention that it is due to prolonged exposure to microorganisms and *nucleases*, further studies are required to investigate if the assertion above will still hold true after a year's period.

The current study did not investigate the underlying factors for the observations made. However, extrapolating from previous studies, the poor performance of the grey-top tubes in Sanger sequencing can be attributed to the presence of sodium fluoride in the vial. Sanger sequencing employs PCR to integrate deoxynucleotides into a growing DNA chain, the mechanism of which is heavily reliant on a magnesium ion (Blanchard *et al.*, 1993). The fluoride anion is known to have a strong interaction with the divalent magnesium cation (Collys *et al.*, 1990). Therefore, aside from fluoride's reported destructive effect on the structure of DNA (Liu *et al.*, 2017), the anion may also interfere with the amplification of DNA through its interaction with the magnesium cation. This supports the argument made above: the lower DNA concentration observed in the purple-top and grey-top tubes before toxicological analysis is partly due to fluoride's and EDTA's interference with the process of quantification. Given the indispensability of magnesium to the process of PCR (Blanchard *et al.*, 1993), the anionic properties of fluoride (Collys *et al.*, 1990), and the known chelating effect of EDTA to metallic ions (Klodos and Skou, 1975), it is reasonable to suggest that this interference occurs at the level of amplification.

4.6. Application of the toxicogenetic assay

The overall metabolic phenotype assigned to the individual whose gene is under examination is dependent on the identified haplotypes. For case 22, the majority of haplotypes suggested a normal (extensive metaboliser) enzyme activity. However, this is heavily impacted by the presence of the *CYP2D6*13* haplotype, which is known to completely eradicate the enzyme's activity (Panserat *et al.*, 1995). The variation in this haplotype occurs at exon 1 of *CYP2D6*, wherein its hybridisation with *CYP2D7* results in the insertion of an additional thymine nucleotide at position 227, thereby causing a shift in the reading frame and a premature termination of translation at amino acid 253 of exon 5 (Panserat *et al.*, 1995). This nullifies any

other haplotype found on the same allele as the *CYP2D6*13* haplotype is located. However, since this SNP was heterozygous in the investigated case, the nullification of enzyme activity caused by the *CYP2D6*13* haplotype does not affect the function of the alternate allele.

Another important haplotype noted was *CYP2D6*86*, whose effect on the activity of the CYP2D6 is not known (Dodgen *et al.*, 2013). If this haplotype exists on the same allele as the *CYP2D6*13* variant, then its effect would be cancelled, as the sequence frameshift caused by *CYP2D6*13* outweighs any single base variation in significance. This would leave the normal activity on the one allele that is not affected by these variants, resulting in an extensive metaboliser phenotype (Sajantila *et al.*, 2010). If, however, the *CYP2D6*86* haplotype exists on a different allele to that of *CYP2D6*13*, the following possible phenotypes exist depending on the effect the variant has on the overall activity of the enzyme: (1) if *CYP2D6*86* confers increased or normal activity, an extensive metaboliser phenotype would result, and (2) if *CYP2D6*86* confers decreased activity, an intermediate metaboliser phenotype would result (Sajantila *et al.*, 2010). In all possible scenarios, the metabolic phenotype can either be extensive metaboliser or intermediate metaboliser. However, since the exact location of the identified variants is not known (as segregation analysis was not performed), the presented phenotypes are merely suggested as the possible phenotypic outcomes based on the possible distribution of the detected SNPs between the two alleles.

Although the lack of segregation analysis (due to no parental samples being available for the study) impeded the ability to ascertain the exact metabolic phenotype of the decedent in question, the experimental question in respect of the current study was successfully answered. All the information that could be collected from Sanger sequencing was successfully obtained. As the success of Sanger sequencing is highly dependent on the quality of DNA, this experiment showed that the blood sample from the red-top tube after toxicological analysis can yield DNA of sufficient quality to perform a toxicogenetic analysis following handling in a toxicology laboratory.

4.7. Strengths and Limitations

Two of the analyses – DNA quantification and purity assessment – performed in this study, showed conclusive results in favour of the grey-top tubes. The assessment of degradation index

did not show one tube type producing better results than another tube type; all the samples met the threshold of non-degraded DNA. Quality assessment by Sanger sequencing was the only experiment in which the red-top tube was shown to produce results favourable over those of the grey-top tubes. This analysis is also a simulation of the intended toxicogenetic analysis; therefore, it holds more weight than any other analysis performed. In other words, while blood stored in grey-top tubes produced DNA samples with higher purity and concentration compared to red-top tubes following toxicological analysis, these were outweighed by the poor performance of the grey-top tubes during sequencing. This study, therefore, demonstrated that DNA concentration and purity cannot be used as proxies for the quality of DNA as far as Sanger sequencing is concerned.

Forensic DNA profiling showed that handling postmortem blood samples in a toxicology laboratory did not result in contamination with extraneous DNA. This, however, is not a guarantee that all blood samples retrieved from a toxicology laboratory in future will be free of extraneous DNA. It is under this premise that we recommend the performance of forensic DNA profiling as a quality assurance step prior to the performance of a toxicogenetic analysis. Also, the study was able to demonstrate the usability of blood retrieved from a toxicology laboratory for a toxicogenetic analysis, as well as highlighting the red-top tubes as best suited to this purpose.

Such information can be utilised for guidance by practitioners (forensic pathologists, toxicologists, and geneticists) involved in postmortem investigation when the need to perform toxicogenetic analysis arises. Therefore, this study provided guidelines around the pre-analytical process of the intended toxicogenetic analysis. Moreover, the availability of the data produced from this study provides an opportunity to the forensic pathology service providers in South Africa to expand the services they are currently rendering. This will ultimately give families more information about the circumstances surrounding the death of their relative, thus minimising the uncertainties that normally accompany cases of sudden unexpected deaths.

However, despite the potentially impactful information that can be drawn from this study, some limitations were noted.

The inclusion criteria that was used to select cases for this study was one that excluded children and cases of immolation and decomposition. This presents a limitation of the application of the designed assay on the blood specimens originating from the aforementioned cases, as the study does not provide evidence showing the viability of such specimens for the assay. Also, the study utilised a sample size of 30 cases to confer statistical significance to the outcome of the research. However, a bigger sample size would be more representative of the wider South African population and give a better indication of how DNA is affected by the handling of blood samples in a toxicological environment.

The extrapolations made for this study were mainly drawn from the information available about the effects of sodium on DNA. To the knowledge of the author, the effects of potassium oxalate on DNA have not been published. This study was not able to answer that question, as the grey-top tube contains both sodium fluoride and potassium oxalate. However, for the purpose of this study, such an analysis was not necessary, as none of the collection tubes used to collect blood specimens for toxicological analysis at Salt River Mortuary contain only potassium oxalate; this additive is always present with sodium fluoride. It suffices to know that collection tubes containing sodium fluoride and potassium oxalate have an effect that renders DNA of poor quality for Sanger sequencing when compared to those that do not contain these additives.

The authors also acknowledge that the differences between the Tox and No-Tox samples were reported as being as a result of handling the other cohort of samples in a toxicological environment. However, it should be noted that DNA was extracted from the Tox samples approximately 16 weeks after blood collection, whereas DNA was extracted from the No-Tox samples within only 72 hours of blood collection. This makes time an undeniable factor in the study. Therefore, it cannot be claimed with certainty that the observed differences between the Tox and No-Tox samples were a result of handling the Tox samples in a toxicological environment, and not a function of time. While the correlation analyses (Appendix C, Section C.3 and Section C.4) showed that the difference in the elapsed time between death and sample collection and that between death and DNA extraction, had no influence on the trends of the data, the current study did not account for the time difference between the post-collection processing of No-Tox samples and that of Tox samples. This can be addressed in further studies by introducing a control sample – duplicate No-Tox samples from which DNA will be extracted at the same time as the Tox samples – to account for the effect of time.

It should be noted, however, that the limitation highlighted above does not nullify the usability of the results of this experiment in the intended toxicogenetic analysis. This is because whether the observed changes were caused by time or exposure to a toxicological environment, both these would still be a factor in real life and the current study was able to show that exposing blood samples stored in red-top tubes to a toxicological environment over a period of 16 weeks does not affect the usability of the DNA for Sanger sequencing. If, in future studies, leaving DNA for longer in postmortem blood samples would be found to be the sole cause of the observed differences between the Tox and No-Tox samples, it would mean that exposing blood samples to a toxicological environment may not have an effect on the quality, concentration, degradation, contamination, and purity of DNA. If, however, handling the samples in a toxicology environment could be found to be an important factor, then this study successfully demonstrated that blood samples collected and stored in red-top tubes are suitable for use in a genetic analysis, following handling involved in the process of toxicological investigation.

4.8. Conclusion

Postmortem blood specimens collected into different types of blood collection tubes (purple-top, grey-top, and red-top tubes) and divided into two groups – samples handled in a toxicological environment and those that were not – underwent various genetic analyses. The red-top tube was shown to be the vial best suited to the storage of blood specimens on which Sanger sequencing will subsequently be performed. The higher DNA concentrations observed in grey-top tubes did not translate into higher quality electropherograms. The lower purity of DNA samples derived from blood handled in a toxicological environment did not impede their usability in DNA sequencing. The limitations of the study were found not to negate the findings made and the application thereof. Overall, blood samples collected and stored in red-top tubes (and not grey-top tubes) over a period of 16 weeks were found to be suitable for use in a genetic analysis, following handling involved in the process of basic toxicological investigation.

CHAPTER 5: REFERENCES

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CHAPTER 6: APPENDICES

APPENDIX A: UCT HREC ETHICS APPROVAL LETTER

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

Room E52-24 Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 404 7682 • Facsimile [021] 406 6411
Email: nosi.tsana@uct.ac.za
Website: www.health.uct.ac.za/fhs/research/humanethics/forms

28 April 2017

HREC REF: 110/2017

Ms L Heathfield
Pathology
Reception, Forensic & Toxicology
Falmouth Building

Dear Ms Heathfield

PROJECT TITLE: POST-MORTEM MOLECULAR AND TOXICOLOGICAL INVESTIGATIONS: EXPLORING TOXICITY AND GENETIC VARIATION IN DECEASED INDIVIDUALS AT SALT RIVER MORTUARY (MPhil-candidate-D Vincent & L Vuko)

Thank you for submitting your study to the Faculty of Health Sciences Human Research Ethics Committee for review.

It is a pleasure to inform you that the HREC has **formally approved** the above-mentioned study.

Approval is granted for one year until the 30th April 2018.

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.
(Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

We acknowledge that the following students Mr D Vincent & L Vuko will be involved in this study.

Please note that for all studies approved by the HREC, the principal investigator **must** obtain appropriate institutional approval before the research may occur.

Please quote the HREC REF in all your correspondence.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, FHS HUMAN RESEARCH ETHICS COMMITTEE
Federal Wide Assurance Number: FWA00001637.

HREC 110/2017

Institutional Review Board (IRB) number: IRB00001938

This serves to confirm that the University of Cape Town Human Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical Research Council (MRC-SA), Food and Drug Administration (FDA-USA), International Convention on Harmonisation Good Clinical Practice (ICH GCP), South African Good Clinical Practice Guidelines (DoH 2006), based on the Association of the British Pharmaceutical Industry Guidelines (ABPI),and Declaration of Helsinki (2013) guidelines.
The Human Research Ethics Committee granting this approval is in compliance with the ICH Harmonised Tripartite Guidelines E6: Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) and FDA Code Federal Regulation Part 50, 56 and 312.

APPENDIX B.1:

INFORMATION FORM AND INFORMED CONSENT FORM

INFORMATION FORM

Study title: Postmortem Toxicogenetics: Determining the suitability of blood samples collected for routine toxicological analyses for use in subsequent genetic analyses.

Researcher: Loyiso Vuko
Supervisor: Ms. Bronwen Davies
Co-supervisors: Laura Heathfield and Ms. Katrina Auckloo

Introduction to the study

You are invited to participate in a research study under University of Cape Town's Division of Forensic Medicine and Toxicology, in the Department of Pathology. It will be conducted by Loyiso Vuko, who is a researcher in the division, and a candidate for an M.Phil degree in Biomedical Forensic Science.

The purpose of this study is to determine whether blood – a biological sample that is routinely collected at autopsy for toxicological analyses (tests that investigate the presence and amount of drugs/chemicals in the body of the deceased) – can be used for a genetic analysis following toxicological analysis. This form explains what you will be asked to do if you decide to participate in this study. Please read it carefully and feel free to ask any questions you may have before you make a decision about participating.

Background

Forensic Pathologists are often faced with cases in which it is not clear from the toxicological analysis performed on samples collected from deceased individuals, whether a death was a result of an intentional or accidental overdose. A test that could be utilised to answer this uncertainty is one that examines if the decedent is not genetically predisposed to experience toxicity from a drug even when taken at normal levels. However, before such a test could be performed, it is important to determine whether the collection tubes into which blood is usually collected at autopsy can keep the blood in a condition that renders it suitable for use in the said genetic test. This is what this study aims to investigate, which would, in turn, inform Forensic Pathologists as to whether or not blood collected into the aforementioned collection tubes can be used for the specified genetic test.

Procedure

You will be asked to give permission to allow for blood to be collected at autopsy by the Forensic Pathologist assigned the case for your deceased family member. The samples will be collected into three different types of collection tubes. Samples collected into the two types of tubes – which are routinely used for toxicological analysis – will undergo toxicological analysis, and the samples collected into the tube custom-made to preserve DNA will not undergo toxicological analysis. DNA will be extracted in all the samples and compared between the different tubes by means of laboratory tests that include spectrophotometry, quantitative polymerase chain reaction and DNA Profiling.

The biological material collected from your deceased family member and the extracted DNA will be stored for a period of 20 years at the Division of Forensic Medicine and Toxicology (authorised institution) at the University of Cape Town. After this time the samples will be appropriately discarded.

The name and every other personal detail of your family member will not be made known. Each case will be given a unique identification code to maintain the anonymity of the deceased. These identification codes will be used to trace the collected samples throughout the course of the research. The confidentiality of the samples and data will be maintained.

The biological samples will not be used for any research unless the research study is reviewed and approved by the University of Cape Town, Faculty of Health Sciences, Human Research Ethics Committee. This committee is responsible for protecting the rights and welfare of individuals who volunteer for participation in research studies.

There are several things you need to know before allowing biological samples to be taken from your deceased family member:

1. When research is carried out, it is not the policy of the University of Cape Town to provide genetic information about the deceased to the family members.

2. Participation in this study is voluntary. You are free not to participate or to withdraw at any time, for whatever reason, without negative consequences. In such cases, no samples will be collected and your choice will not affect the way you will be treated at the Salt River Mortuary.

3. If you participate in the study, you can change your mind later and decide that you don't want to participate anymore and you do not want the tissue to be used in this study. Please let us know and we will destroy the samples. If the sample has already been analysed at the time you change your mind, your results and other data may have already been shared with other investigators. In that case, we will not be able to destroy this data. The data will be removed from the secured database. That means that no additional researchers can get the data.

4. You will not receive feedback of possible genetic variations that are found in the DNA of the deceased. This study is unlikely to benefit you or your family directly, but it is hoped that it will contribute to knowledge about the influence of genes in drug-toxicity in the future.

5. There will be no cost to you and there will be no compensation for your participation.

Making your choice

Please read each sentence below and think about your choice. After reading each sentence, please tick the Yes or No box. No matter what you decide, it will not negatively affect you or your deceased family member in any way.

If you may have any questions or require referral to a grief center or psychological support, please don't hesitate to ask the person taking the consent. If you may have any questions with regards to the rights and welfare of a research subject in the study, please contact the Chairperson of the University Of Cape

Town Faculty Of Health Science Human Research Ethics Committee, **Professor Marc Blockman** on (021) 406 6496. If you require any further information about this study, please contact Laura Heathfield: (0212) 406 6569 or laura.heathfield@uct.ac.za.

If the spouse/partner/major child/parent/guardian/major sibling agrees then the consent form needs to be read and informed consent will be taken. **Please note that the information and consent forms will be translated into the family member's language of choice.**

- Thank you for your time -

Please find attached the consent form to be signed if you wish to proceed

CONSENT FORM

I, _____ (next-of-kin, full name), the spouse/partner/major child/parent/guardian/major brother/major sister (circle relationship) of the deceased -

1. Confirm that I have:	**Yes**	**No**
a) Read and understood contents of this form, been informed about this study's purpose, procedures, possible benefits, and risks, and agree to be a part of the research study.		

2. Give consent and agree that:	**Yes**	**No**
a) Blood sample(s) can be taken from my deceased family member and subjected to laboratory tests at the Division of Forensic Medicine and Toxicology		
b) The blood samples and extracted DNA may be stored for a period of 20 years after which it will be appropriately discarded.		
c) The stored blood and genetic material may only be used for further research which have been reviewed and approved by the University of Cape Town, Faculty of Health Sciences Human Research Ethics Committee.		
d) I may be contacted in the future by someone from the University of Cape Town who may ask me to take part in research that may develop from the results of the study.		

3. Further understand that:	**Yes**	**No**
a) The treatment and management of the biological samples of my deceased family member will be in accordance with the guidelines of the University Of Cape Town Faculty Of Health Science Human Research Ethics Committee.		
b) This study is unlikely to benefit me or my family directly.		
c) I can, at any time, withdraw my consent and that I have to notify the primary investigator of my decision to withdraw.		

I have explained to _____(full name) who is the spouse/partner/major child/parent/guardian/major brother/major sister of the deceased; the purpose, procedures, possible benefits and discomfort of this research study; and how the samples will be collected and stored for use in the study and in possible further research by other individuals within the Division of Forensic Medicine and Toxicology at the University of Cape Town.

Full name of person obtaining consent

Signature of person obtaining consent Date

Full name of person authorising consent for collection of samples at autopsy for use in research study

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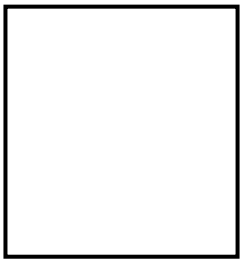
Signature of person authorising consent for collection of samples at autopsy for use in research study Date

Thumb print of the next-of-kin of the deceased:



Full name of witness

Signature of witness Date

ISIHLOMELO B.2:

IFOMU YOLWAZI KUNYE NEFOMU YEMVUME ESEKELWE ELWAZINI

IFOMU YOLWAZI

Isihloko sophando: *Postmortem Toxicogenetics*: Ukufumanisa ukufaneleka kwegazi elisetyenziswa ngokusesikweni kuhlalutyo lwe*toxicology* ekusetyenzisweni kuvavanyo lwemfuza.

Umphandi: Mnu. Loyiso Vuko
Umphathi: Nksk. Bronwen Davies
Usekela-mphathi: Nksk. Laura Heathfield kunye noNksk. Kathrina Auckloo

Intshayelelo yophando

Uyamenywa ukuba athathe inxaxheba kwisifundo sophando esiphantsi kweCandelo le*Forensic Medicine* ne*Toxicology*, kwiSebe le*Pathology*, eYunivesithi yaseKapa. Luya kuqhutywa nguLoyiso Vuko, ongumphandi kwicandelo, kwaye ongumviwa kwisidanga se*M.Phil* esifundweni se*Biomedical Forensic Science*.

Injongo yolu phando kukuqinisekisa ukuba igazi – lona elisetyenziswayo ngokwesiqhelo kuvavanyo lwe*toxicology* (uhlalutyo oluyindlela yokuphanda ubukho kunye nesixa seziyobisi emzimbeni womntu) – lingasetyenziswa kuhlalutyo lwemfuza (*genetics*) emva kohlalutyo lwe*toxicology* olwenziwa kumfi. Le fomu icacisa ukuba uza kucelwa ntoni xa uthathe isigqibo sokuba uzathatha inxaxheba kolu phando. Nceda uyifunde ngononophelo kwaye uzive ukhululekile ukubuza nayiphi na imibuzo onokuba nayo ngaphambili kokuba uthathe isigqibo malunga nokuthatha inxaxheba.

Imvelaphi yophando

Ogqirha bakwa*Forensic Pathology* badla ngokujongana nemeko apho kungacaciyo kutyando olwenziwayo kubantu abangasekhoyo ukuba ukusweleka komfi kubangelwe kukuthatha iziyobisi/amachiza ngaphezulu komlinganiselo ngenjongo okanye ngempazamo. Uvavanyo olinokuthi lusetyenziswe ukuphendula esisehlo loluphononong a ukuba umfi akanafuzo olumenza atyhefeke lichiza naxa elithathe ngokomlinganiselo. Nangona kunjalo, phambi kokuba uvavanyo olululuhlobo lwenziwe, kubalulekile ukuqinisekisa ukuba izikhongozeli ezidla ngokusetyenziswa ukuqokelela igazi kolu tyando ziyakwazi ukugcina igazi likwimeko efanelekileyo ukuze lisetyenziswe koluvavanyo lwemfuza. Esi sifundo sijolise ekuphandeni lonto, yaye iziphumo ziyakuthi zazise oogqirha bakwa*Forensic Pathology* ukuba igazi eliqokelelwe kwezizikhongozeli ezikhankanyiweyo lingasetyenziswa koluvavanyo lwemfuza osoluxeliweyo.

Inkqubo yophando

Uya kucelwa ukuba unike imvume yokuba igazi liqokelelwe xa kusenziwa utyando kwilungu losapho lwakho ngugqirha. Eli gazi lizakufakwa kwiintlobo ezintathu ezahlukileyo zezikhongozeli zokuqokelela igazi. Kwiintlobo ezimbini zezikhongozeli kuza kufakwa igazi elizakusetyenziswa ukuphanda amachiza okanye iziyobisi ezisegazini lomfi kuhlalutyo lwe*toxicology*. Igazi elifakwe kweyesithathu intlobo yesikhongozeli – esisetyenziswa ngokusesikweni ukulondoloza i*DNA* – alizukusetyenziswa koluhlalutyo lwe*toxicology*. Emveni koko, i*DNA* iya kuthathwa kuwo onke amagazi kwaye ithelekiswe phakathi kwezikhongozeli ezahlukileyo ngokusebenzisa iimvavanyo eziquka i*spectrophotometry*, i*quantitative Polymerase Chain Reaction* kunye ne*DNA Profiling*.

Igazi kunye ne*DNA* ezithathwe kwilungu elingasekhoyo losapho lwakho liya kugcinwa isithuba seminyaka angama-20 kwiCandelo le*Forensic Medicine* ne*Toxicology* (iziko eligunyazisiweyo), eYunivesithi yaseKapa. Emva kwesi sithuba le*DNA* iya kutshatyalaliswa ngokufanelekileyo.

Igama kunye nezinye iinkcukacha zobuqu zelungu losapho lwakho aziyi kwaziswa. Ityala ngalinye liya kunikwa isazisi esahlukileyo ukufihla iinkcukacha zomfi. Esi sazisi siya kusetyenziselwa ukulandelela igazi lomfi lude lufikele esiphelweni uphando.

Igazi elitsaliweyo aliyi kusetyenziselwa naluphi na uphando ngaphandle kophando oluye lwaphononongwa ngokutsha lamkelwa yi*Human Research Ethics Committee* yaseYunivesithi yaseKapa. Le komiti inoxanduva lokukhusela amalungelo kunye nentlalontle yabantu abathatha inxaxheba kuphando.

Izinto ekufuneka uzazi phambi kokuba uvume ukuba igazi litsalwe kwilungu elingasekhoyo losapho lwakho zezi:

6. Xa uphando luqhuba, umgaqo siseko weYunivesithi yaseKapa awuvumi ukuba amalungu osapho aziswe malunga nemfuza yalowo ungasekhoyo.

7. Ukuthatha inxaxheba kolu phando akusosinyanzeliso. Ukhululekile ukuba wale okanye urhoxise isigqibo sakho nangaliphi na ixesha, ngenxa yaso nasiphi na isizathu. Kwiimeko ezinjalo, igazi aliyi kutsalwa kwaye ukhetho lwakho aluyi kuchaphazela indlela ophathwa ngayo kwi*mortuary* yaseSalt River.

8. Ukuba uthathe inxaxheba kolu phando, ungaphinde utshintshe ingqondo yakho, urhoxise isigqibo sakho. Nceda usazise xa kunjalo ukwenzele sizo kutshabalalisa igazi elitsaliweyo. Ukuba igazi sele lisetyenzisiwe ngexesha otshintsha ingqondo ngalo, kungenzeka ukuba iziphumo zalo kunye nezinye iinkcukacha sele kwabelwene ngazo nabanye abaphandi. Xa kunjalo, asiyi kuba nako ukurhoxisa ezoziphumo. Kodwa ezo nkcukacha ziya kususwa kuvimba okhuselekileyo. Oko kuthetha ukuba abekho abanye abaphandi abazakwazi ukuzifumana iziphumo zegazi lelungu losapho lwakho.

9. Awuyi kufumana ngxelo ngenkcukacha zemfuza ezifumaneka kwi*DNA* yomfi. Olu phando alunayo inzuza eqonde wena okanye usapho lwakho ngqo, kodwa sinethemba lokuba iziphumo zalo ziya kuba negalelo kulwazi malunga nempembelelo yemfuza ekutyhefekeni.

10. Akuyi kubakho zindleko okanye imbuyekezo ngokuthatha inxaxheba koluphando.

Ukwenza ukhetho lwakho

Nceda ufunde isivakalisi ngasinye apha ngezantsi ulandelise ngokucinga ukhetho lwakho. Emva kokufunda isivakalisi ngasinye, nceda uphawule Ewe okanye Hayi kwibhokisi oyinikiweyo. Isigqibo sakho asiyi kuchaphazela wena okanye ilungu elingasekhoyo losapho lwakho nangayiphi na indlela.

Ukuba unokuba nayiphi na imibuzo okanye ufuna ukuthunyelwa kwiziko elijongene nabo basentlungwini okanye inkxaso ngokwasengqondweni, nceda ungathandabuzi ukubuza umntu othatha lemvume. Ukuba unokuba nayiphi na imibuzo malunga namalungelo kunye nentlalontle yalowo uthatha inxaxheba kuphando, nceda uqhagamshelane noSihlalo we*Human Research Ethics Committee* yaseYunivesithi yaseKapa kwi*Faculty* ye*Health Science*, u**Njingalwazi Marc Blockman** ku (021) 406 6496. Ukuba ufuna nayiphi na ingcaciso engaphezulu ngesi sifundo, nceda uqhagamshelane noLaura Heathfield: (0212) 406 6569 okanye laura.heathfield@uct.ac.za.

Ukuba umlingane/iqabane/umntwana omdala/umzali/umgcini/inkulu yekhaya iyayinikeza imvume, ifom yemvume kufuneka ifundwe phambi kokuba kuthatyathwe imvume. **Nceda uqaphele ukuba le ngcaciso kunye nalemvume ziza kuguqulelwe kulwimi olukhethileyo.**

- Enkosi ngexesha lakho -

Nceda ufumane ifomu eqhotyoshelweyo uze ulandelise ngokuyityikityele ukuba unqwenela ukuqhubeka

IFOMU YEMVUME

Mna, _____ (igama elipheleleyo lesalamane), umlingane/iqabane/umntwana omdala/umzali/umgcini/ubhuti omdala/ udade omdala womfi –

4. Ndiyaqinisekisa ukuba:	**Ewe**	**Hayi**
b) Ndikufundile ndakuqonda okubhalwe kule fomu, ndazisiwe ngenjongo, iinkqubo, imivuzo, neengozi zoluphando, yaye ndiyavuma ukuba yinxalenye yesi sifundo.		

5. Ndinika imvume kwaye ndiyavuma ukuba:	**Ewe**	**Hayi**
e) Igazi lingatsalwa kwilungu elingasekhoyo losapho lwam kwaye livavanywe elebhu kwiCandelo le*Forensic Medicine* ne*Toxicology*.		
f) Igazi ne*DNA* ezitsaliweyo zingagcinwa isithuba seminyaka angama-20 emva koko zizakutshatyalaliswa ngokufanelekileyo.		
g) Igazi eligciniweyo ne*DNA* zingasetyenziswa kuphando oluye laphononongwa lamkelwa yi*Human Research Ethics Committee* yaseYunivesithi yaseKapa kuphela.		
h) Ndingaqhagamshelwa kwixesha elizayo ligosa laseYunivesithi yaseKapa licele ukuba ndithathe inxaxheba kwisifundo esiphuhlisa iziphumo zoluphando.		

6. Ngaphezu koko, ndiyaqonda ukuba:	**Ewe**	**Hayi**
d) Impatho nolawulo lwegazi lelungu losapho lwam iya kuhambelana nezikhokelo ze*Human Research Ethics Committee* yaseYunivesithi yaseKapa.		
e) Olu phando aluyi kuba namvuzo kum okanye usapho lwam ngqo.		
f) Ndingakwazi, nangaliphi na ixesha, ukurhoxisa imvume yam yaye kufuneka ndazise umphandi ophambili ngesigqibo sam sokurhoxa.		

Ndicacisile ku _____(igama elipheleleyo) ongumlingane/iqabane/umntwana omdala/umzali/umgcini/ubhuti omdala/ udade omdala womfi; injongo, iinkqubo, imivuzo nobungozi boluphando; indlela elizathathwa ligcinwe ngayo igazi ukuze lisetyenziswe kwesisifundo nakwezinye kwixesha elizayo ezizakuqhutywa ngabaphandi abaphantsi kweCandelo le*Forensic Medicine* ne*Toxicology*, kwiSebe le*Pathology*, eYunivesithi yaseKapa.

Igama elipheleleyo lomntu uthatha imvume

Utyikityo lomntu othatha imvume Umhla

Igama elipheleleyo lomntu ogunyazisa imvume yokuthathwa kwegazi elizakusetyenziswa kuphando

Utyikityo lomntu ogunyazisa imvume Umhla
yokuthathwa kwegazi elizakusetyenziswa kuphando

Ushicilelo lukabhontsi wesalamane salowo ungasekhoyo:

Igama elipheleleyo lengqina

Utyikityo lwengqina Umhla

ADDENDUM B.3:

INFORMASIE VORM EN INGELIGTE TOESTEMMING VORM

INFORMASIE VORM

Studie Titel: Nadoodse Toksiko-genetika: Geskiktheid bepaling van bloed monsters ingesamel tydens roetine toksikologiese ontleding vir die gebruik in daaropvolgende genetiese analise.

Navorser: Mnr. Loyiso Vuko
Studieleier: Me. Bronwen Davies
Mede-studieleiers: Me. Laura Heathfield en Me. Katrina Auckloo

Inleiding tot die studie

U word genooi om deel te neem aan ’n navorsingstudie by die Universiteit van Kaapstad, Divisie van Forensiese Medisyne en Toksikologie, Departement van Patologie. Die navorsing word onderneem deur Loyiso Vuko, ’n navorser in die divisie en ’n kandidaat vir ’n MPhil graad in Biomediese Forensiese Wetenskap.

Die doel van hierdie studie is om te bepaal of bloed, ’n biologiese monster wat normaalweg geneem word tydens ’n nadoodse ondersoek vir toksikologiese ontledings (toetse wat die teenwoordigheid en hoeveelheid van sekere dwelms/chemikalieë/terapeutiese middels in die liggaam van die oorledene ondersoek) – gebruik kan word vir genetiese analise wat volg op die toksikologiese toetse. Hierdie vorm verduidelik wat van u gevra sal word as u besluit om deel te neem in die navorsing. Lees asseblief die informasie hieronder aandagtig en bespreek gerus enige vrae of bekommernisse voor u besluit om deel te neem.

Agtergrond

Forensiese Patoloë is gereeld betrokke in gevalle waar daar onduidelikheid is aangaande die toksikologiese resultate van monsters geneem tydens outopsie, spesifiek die onderskeid tussen ’n opsetlike oordosis of ’n toevallige/ongeluk oordosis. Een manier wat gebruik kan word om hierdie onduidelikheid aan te spreek is om te bepaal of die oorledene geneties ingestel is om toksiese vlakke van ’n middel te ervaar, alhoewel ’n normale dosis geneem was. Daar moet egter eers bepaal word of die versameling buise waarin die bloedmonsters geneem word, geskik is vir genetiese toetse. Die voorgenoemde stelling is die vraagstuk vir hierdie navorsing en sal in die toekoms Forensiese Patoloë kan inlig of hierdie buise geskik is vir genetiese toetse.

Prosedure

U sal gevra word om toestemming te gee vir die insameling van ’n bloedmonster tydens outopsie van u oorlede familielid. Die Forensiese Patoloog toegeken aan die saak sal die bloedmonster neem. Drie verskillende buise sal gebruik word vir die insameling. Twee van hierdie buise is roetine toksikologiese buise en sal toksikologiese ontleding ondergaan. Die derde tipe buis preserveer DNS en sal nie toksikologiese ontleding ondergaan nie. DNS sal onttrek word vanuit al die bloedmonsters en vergelyk word op grond van verskillende tipes laboratorium toetse. Hierdie toetse sluit in; spektrofotometrie, kwantitatiewe polimerase ketting reaksie en DNS profilering.

Die bloedmonster geneem van u familielid én die DNS onttrek uit die monster sal vir 20 jaar gestoor word in die Divisie van Forensiese Medisyne en Toksikologie (gemagtigde instansie) by die Universiteit van Kaapstad. Na hierdie tydperk sal die monsters op ŉ toepaslike manier vernietig word.

Die naam van u familielid, asook enige ander persoonlike inligting, sal op geen manier bekend gemaak word nie. Elke geval word ŉ unieke studie kode gegee om anonimiteit te behou. Hierdie kode word slegs gebruik om die monsters te spoor tydens die leeftyd van die navorsing. Die vertroulikheid van alle monsters en data sal ten alle tye behou word.

Die monsters sal nie vir navorsing gebruik word, tensy die navorsing hersien en goedgekeur word deur die Universiteit van Kaapstad, Fakulteit Gesondheidswese, Menslike Navorsing Etiek Komitee. Dié komitee is verantwoordelik vir die beskerming van die regte en welsyn van individue wat deel neem aan navorsing.

Daar is verskeie aspekte wat u uself moet van verwittig voor u instem dat biologiese monsters geneem mag word van u oorlede familielid:

1. Wanneer navorsing onderneem word, is dit nie die beleid van die Universiteit van Kaapstad dat enige genetiese informasie teruggee word aan familielede nie.

2. Deelname aan hierdie navorsing is vrywillig. U is gemagtig om te verkies dat u familielid nie deel vorm van die studie nie, asook kan u enige tyd verkies om te onttrek uit die studie vir enige rede sonder gevolge. In só ŉ geval sal geen monsters geneem word nie en u keuse sal onder geen omstandighede affekteer hoe u behandel word by ‘Salt River Mortuary’ nie.

3. As u nou instem vir deelname in die navorsing maar later besluit om te onttrek moet u asseblief die navorser laat weet. In só ŉ geval sal die biologiese materiaal vernietig word. As die biologiese monster reeds analise ondergaan het, mag die resultate van die analise moontlik al reeds gedeel wees met ander navorsers. Dus sal die data nie ten volle verwyder kan word nie. Alhoewel, die data sal verwyder word van die databasis sodat geen addisionele navorsers die data sal kan ontleed nie.

4. U sal nie terugvoer ontvang van enige genetiese mutasies gevind in die DNS van u familielid nie. Dit is onwaarskynlik dat die studie, u of u familie direk sal bevoordeel. Dit word egter beoog dat die navorsing sal bydra tot ons kennis aangaande die invloed van gene in dwelm-toksisiteit.

5. Daar is geen koste gebonde aan deelname in die navorsing nie. U sal ook geen vergoeding ontvang vir deelname nie.

Om u keuse te maak

Lees asseblief elke stelling hieronder aandagtig voor u antwoord. Na u elke stelling gelees het, merk asseblief die ‘Ja’ of ‘Nee’ boksie. Ongeag wat u antwoord is op enige van die stellings, dit sal nie u, of u familielid, negatief beïnvloed nie.

As u enige navrae het, of u benodig verwysing na ŉ beradingsentrum of psigologiese ondersteuning, moet asseblief nie huiwer om u vraag te rig aan die persoon wat hierdie vorm

bespreek nie. As u enige navrae het aangaande die regte en welsyn van individue in navorsing, kontak gerus die voorsitter van die Universiteit van Kaapstad, Fakulteit Gesondheidswese, Menslike Navorsing Etiek Komitee, **Professor Marc Blockman** op (021) 406 6496. As u enige verdere inligting benodig aangaande die studie onderwerp, kontak gerus vir Laura Heathfield op (021) 406 6569 of laura.heathfield@uct.ac.za.

Indien die eggenoot/vennoot/kind/ouer/voog/broer/suster in stem, moet die ingeligte toestemmingsvorm gelees en ingevul word. **Neem asseblief kennis dat die toestemmingsvorm vertaal sal word in die verkose taal van die naasbestaande.**

-U word bedank vir u tyd -

Vind asseblief hier aangeheg die toestemmingsvorm as u sou wens om voort te gaan

TOESTEMMINGSVORM

Ek,_____
(naasbestaande, volle name), die eggenoot/vennoot/kind/ouer/voog/broer/suster (omsirkel verhouding) van die oorledene -

7. Bevestig dat ek:	**Ja**	**Nee**
c) Die inhoud van hierdie vorm gelees en verstaan het, dat ek ingelig is aangaande die doel van die navorsing, enige prosedures, voordele en gevare en ek gee my toestemming vir deelname in die studie.		

8. Gee toestemming en stem in dat:	**Ja**	**Nee**
i) Bloedmonsters van my oorlede familielid mag geneem word en onderwerp word aan laboratorium toetse by die Divisie van Forensiese Medisyne en Toksikologie.		
j) Die bloedmonsters en DNS gestoor mag word vir ’n tydperk van 20 jaar waarna dit toepaslik sal vernietig word.		
k) Die gestoorde bloed en genetiese materiaal net vir verdere navorsing gebruik mag word indien dié navorsing hersien en goedgekeur word deur die Universiteit van Kaapstad, Fakulteit Gesondheidswese, Menslike Navorsing Etiek Komitee.		
l) Ek mag gekontak word in die toekoms deur die Universiteit van Kaapstad wat my mag vra om deel te neem in navorsing wat moontlik kan ontwikkel deur die resultate van die huidige navorsing.		

9. Verstaan verder dat:	**Ja**	**Nee**
g) Die behandeling en bestuur van die biologiese monsters van my oorlede familielid sal geskied in oorstemming met die riglyne van die Universiteit van Kaapstad, Fakulteit Gesondheidswese, Menslike Navorsing Etiek Komitee.		
h) Dit is onwaarskynlik dat die studie my of my familie direk sal bevoordeel.		
i) Ek mag enige tyd my toestemming onttrek en ek moet die hoof navorser in kennis stel aangaande só ’n besluit.		

Ek het verduidelik aan _____ (volle name), die eggenoot/vennoot/kind/ouer/voog/broer/suster van die oorledene; die doel van die navorsing, enige prosedures, voordele en gevare; hoe die bloedmonsters geneem en gestoor sal word tydens hiérdie navorsing en moontlike toekomstige navorsing deur ander navorsers by die Divisie van Forensiese Medisyne en Toksikologie, Universiteit van Kaapstad.

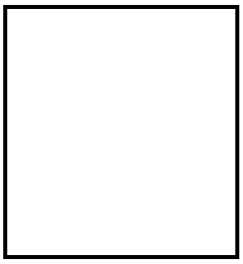
Volle name (Persoon wat toestemming verkry)

Handtekening (Persoon wat toestemming verkry) Datum

Volle name (Naasbestaande)

Handtekening (Naasbestaande) Datum

Duim afdruk van naasbestaande:



Volle name (Getuie)

Handtekening (Getuie) Datum

APPENDIX C: SUPPLEMENTARY DATA

C.1. DNA Quantification by spectrophotometry.

To see if the trends seen for the concentrations obtained by qPCR were reproduced in an analytical technique with a different principle, DNA concentrations were also assessed using spectrophotometry, where an average concentration of elution 1 and elution 2 was calculated for each tube. The distributions and median concentrations for all the sample types are shown in Figure S1.

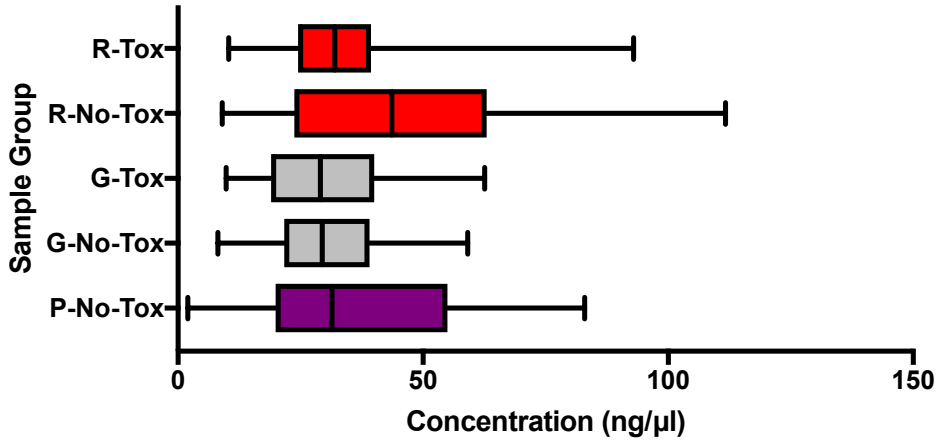


Figure S1: DNA concentration assessed by spectrophotometry. Postmortem blood samples (4 mL) were collected into three types of blood collection tubes: purple-top (P), grey-top (G), and red-top (R) tubes. The blood samples were stored at 4˚C and DNA was isolated within 72 hours of collection from the No-Tox samples. The G-Tox and R-Tox tubes were handled in a toxicological environment (at 4˚C) and DNA was extracted approximately 16 weeks after blood collection. DNA was extracted using the QiaAmp DNA Investigator's Kit (Qiagen, Hilden, Germany) and NanoDrop 2000 (Thermo Scientific, Massachusetts, USA) was used to quantitate the isolated DNA on the day of its extraction.

The concentrations obtained by spectrophotometry were generally higher (average = 32.3 ng/µL) than those obtained by qPCR (average = 20.0 ng/µL). As was the case with qPCR concentrations (Figure 1), the R-No-Tox group produced the highest median concentration of 43.6 ng/µL. However, contrary to what was seen in qPCR analysis, the R-Tox group produced the second highest median concentration of 32.0 ng/µL. This also means that, in the Tox inter-tube type analysis, the G-Tox group had a lower median concentration (29.1 ng/µL), which contradicts the qPCR results (Figure 1). It was also noted that the G-Tox medial concentration was similar to that of the grey-top tubes that were not handled in a toxicological environment (29.4 ng/µL). The purple-top group had the lowest median concentration of 27.3 ng/µL.

C.2. Assessment of the quality of DNA by Sanger sequencing

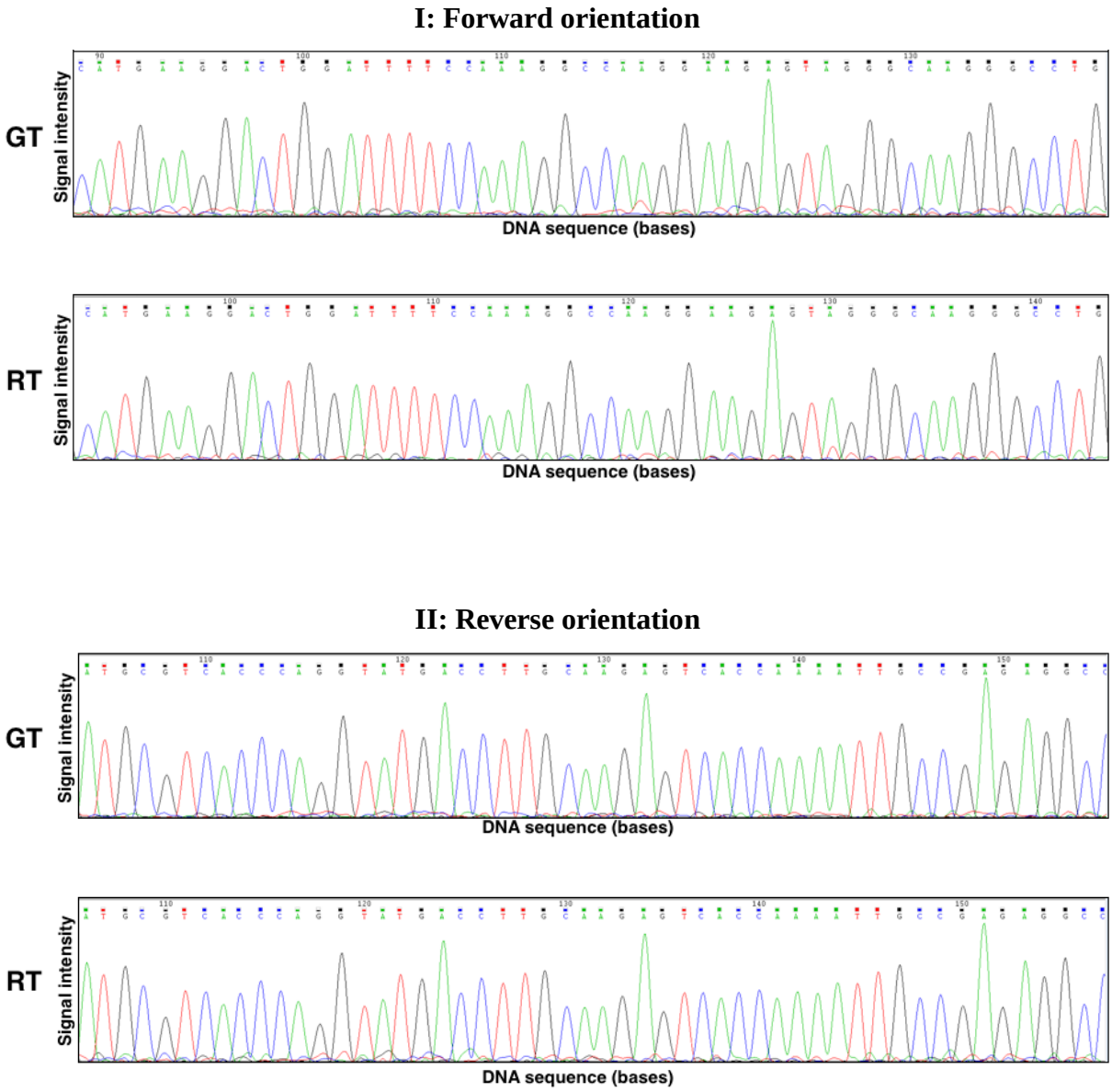


Figure S2: **A portion of the DNA sequence of target B**. Postmortem blood samples (4 mL) were collected into two types of blood collection tubes: grey-top (GT) and red-top (RT) tubes. The blood samples were handled in a toxicological environment (at 4˚C) and DNA was isolated approximately 16 weeks after collection using the QiaAmp DNA Investigator's Kit (Qiagen, Hilden, Germany). The targets of *CYP2D6* were amplified by PCR using primers designed in-house. The sequences of the (I) forward complement and the (II) reverse complement of target B were determined by a Sanger sequencing method that utilised the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA).

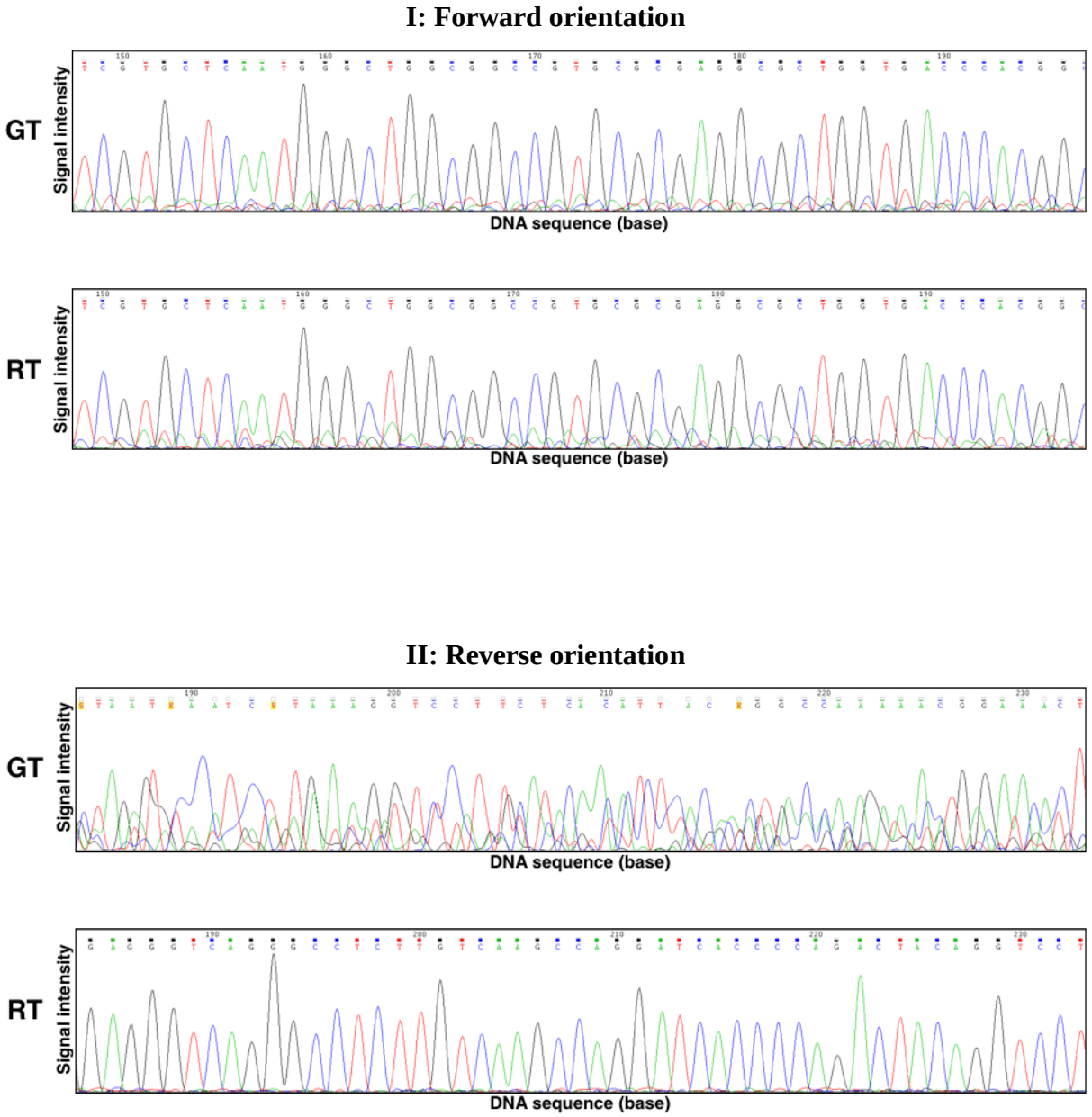


Figure S3: **A portion of the DNA sequence of target C**. Postmortem blood samples (4 mL) were collected into two types of blood collection tubes: grey-top (GT) and red-top (RT) tubes. The blood samples were handled in a toxicological environment (at 4˚C) and DNA was isolated approximately 16 weeks after collection using the QiaAmp DNA Investigator's Kit (Qiagen, Hilden, Germany). The targets of *CYP2D6* were amplified by PCR using primers designed in-house. The sequences of the (I) forward complement and the (II) reverse complement of target C were determined by a Sanger sequencing method that utilised the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA).

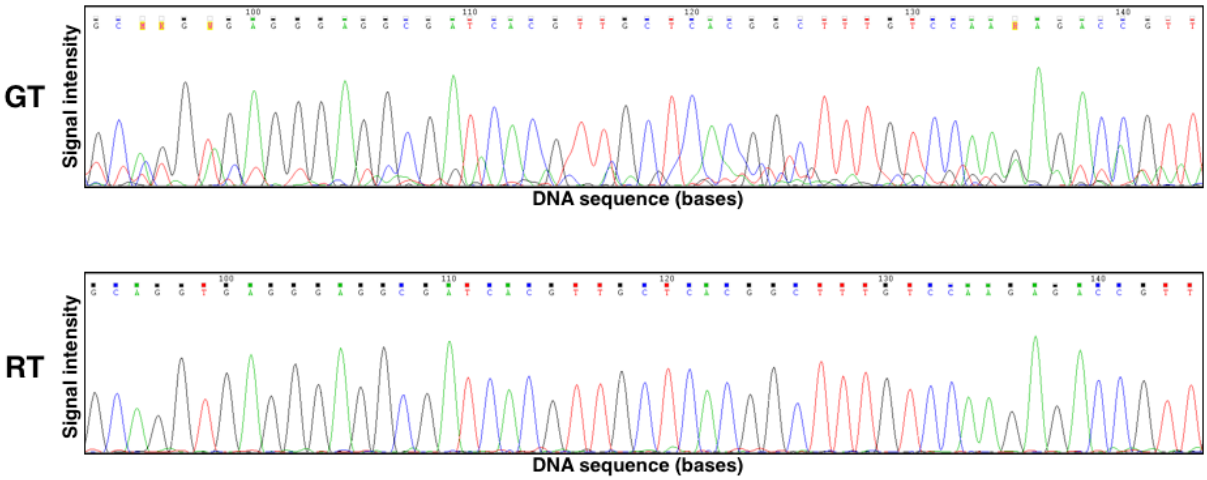


Figure S4: **A portion of the DNA sequence of target D**. Postmortem blood samples (4 mL) were collected into two types of blood collection tubes: grey-top (GT) and red-top (RT) tubes. The blood samples were handled in a toxicological environment (at 4˚C) and DNA was isolated approximately 16 weeks after collection using the QiaAmp DNA Investigator's Kit (Qiagen, Hilden, Germany). The targets of *CYP2D6* were amplified by PCR using primers designed in-house. The sequence of the reverse complement of target D was determined by a Sanger sequencing method that utilises the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA).

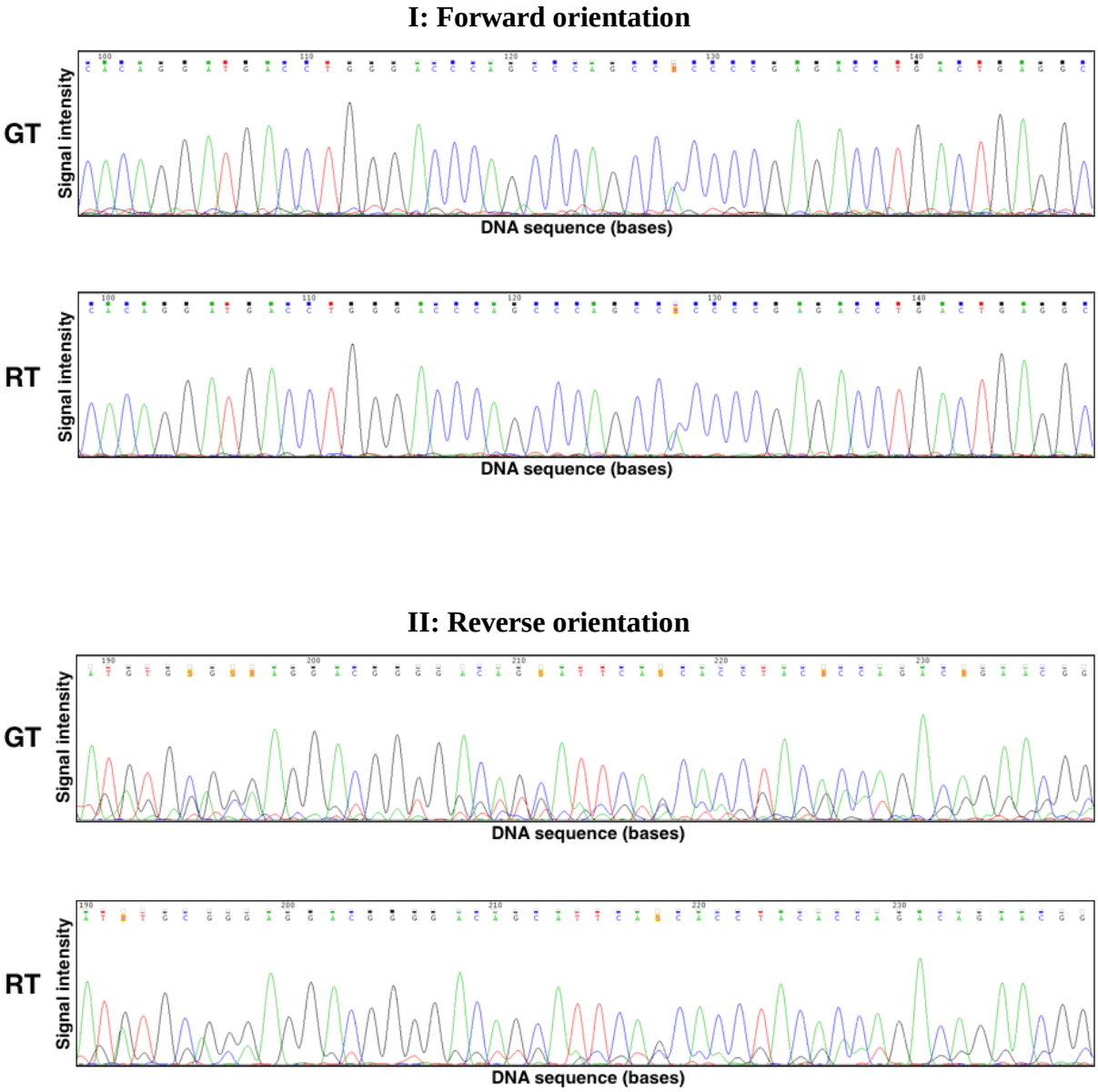


Figure S5: **A portion of the DNA sequence of target E**. Postmortem blood samples (4 mL) were collected into two types of blood collection tubes: grey-top (GT) and red-top (RT) tubes. The blood samples were handled in a toxicological environment (at 4˚C) and DNA was isolated approximately 16 weeks after collection using the QiaAmp DNA Investigator's Kit (Qiagen, Hilden, Germany). The targets of *CYP2D6* were amplified by PCR using primers designed in-house. The sequences of the (I) forward complement and the (II) reverse complement of target E were determined by a Sanger sequencing method that utilised the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA).

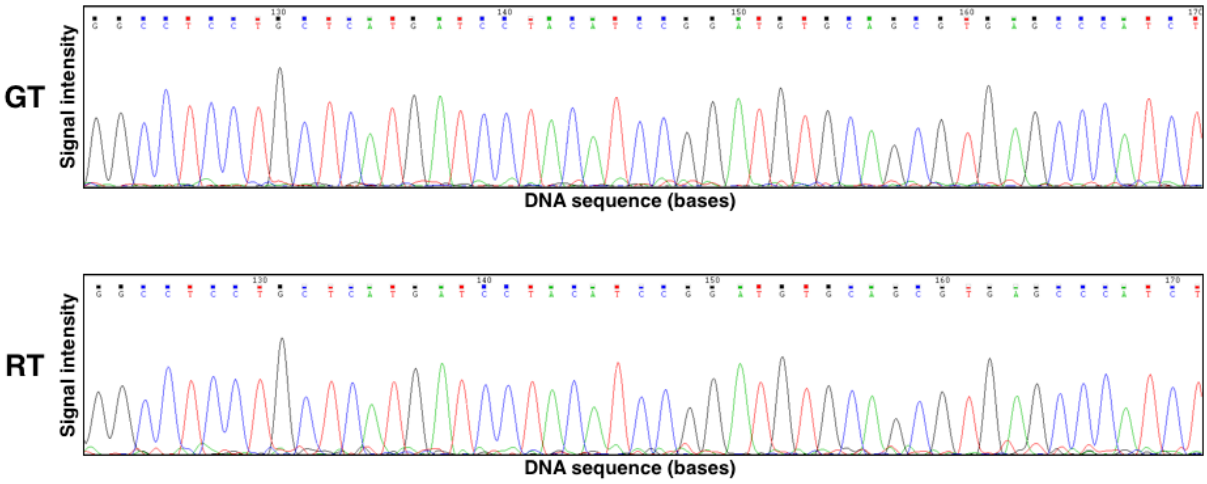


Figure S6: A portion of the DNA sequence of target F. Postmortem blood samples (4 mL) were collected into two types of blood collection tubes: grey-top (GT) and red-top (RT) tubes. The blood samples were handled in a toxicological environment (at 4˚C) and DNA was isolated approximately 16 weeks after collection using the QiaAmp DNA Investigator's Kit (Qiagen, Hilden, Germany). The targets of *CYP2D6* were amplified by PCR using primers designed in-house. The sequence of the forward complement of target F was determined by a Sanger sequencing method that utilises the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA).

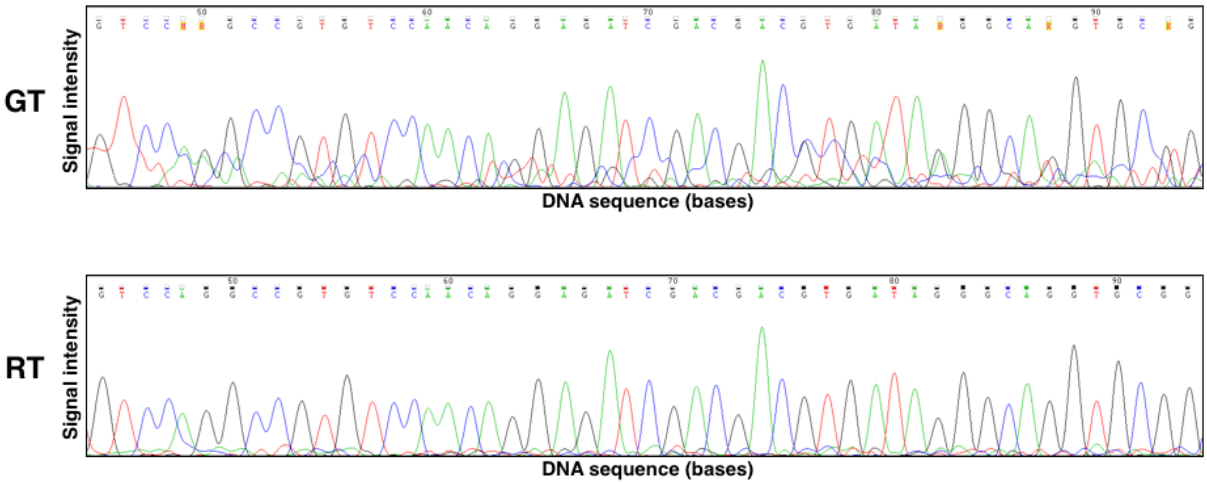


Figure S7: A portion of the DNA sequence of target G. Postmortem blood samples (4 mL) were collected into two types of blood collection tubes: grey-top (GT) and red-top (RT) tubes. The blood samples were handled in a toxicological environment (at 4˚C) and DNA was isolated approximately 16 weeks after collection using the QiaAmp DNA Investigator's Kit (Qiagen, Hilden, Germany). The targets of *CYP2D6* were amplified by PCR using primers designed in-house. The sequence of the forward complement of target G was determined by a Sanger sequencing method that utilised the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA).

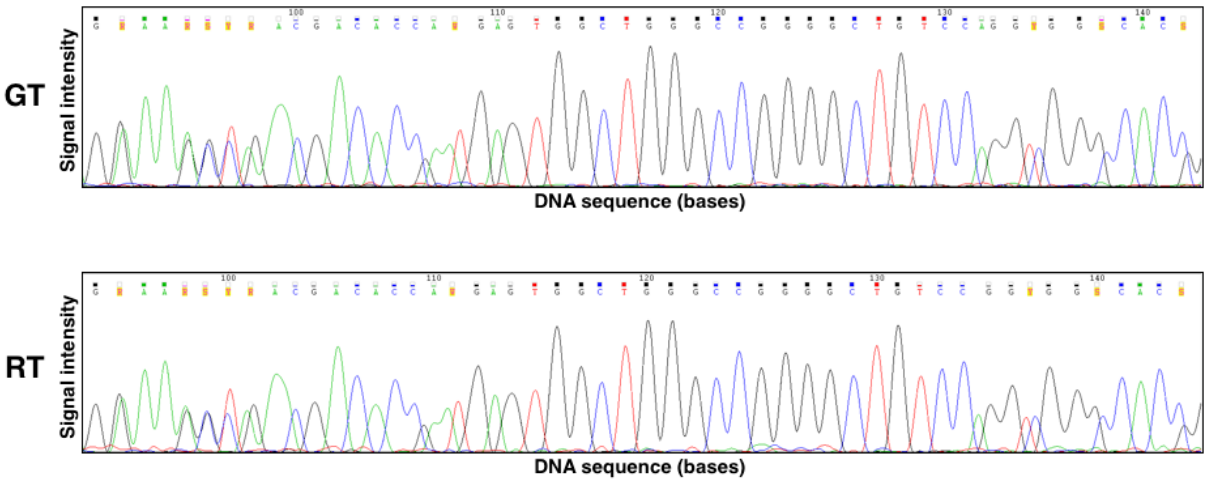


Figure S8: A portion of the DNA sequence of target H. Postmortem blood samples (4 mL) were collected into two types of blood collection tubes: grey-top (GT) and red-top (RT) tubes. The blood samples were handled in a toxicological environment (at 4˚C) and DNA was isolated approximately 16 weeks after collection using the QiaAmp DNA Investigator's Kit (Qiagen, Hilden, Germany). The targets of *CYP2D6* were amplified by PCR using primers designed in-house. The sequence of the reverse complement of target H was determined by a Sanger sequencing method that utilised the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA).

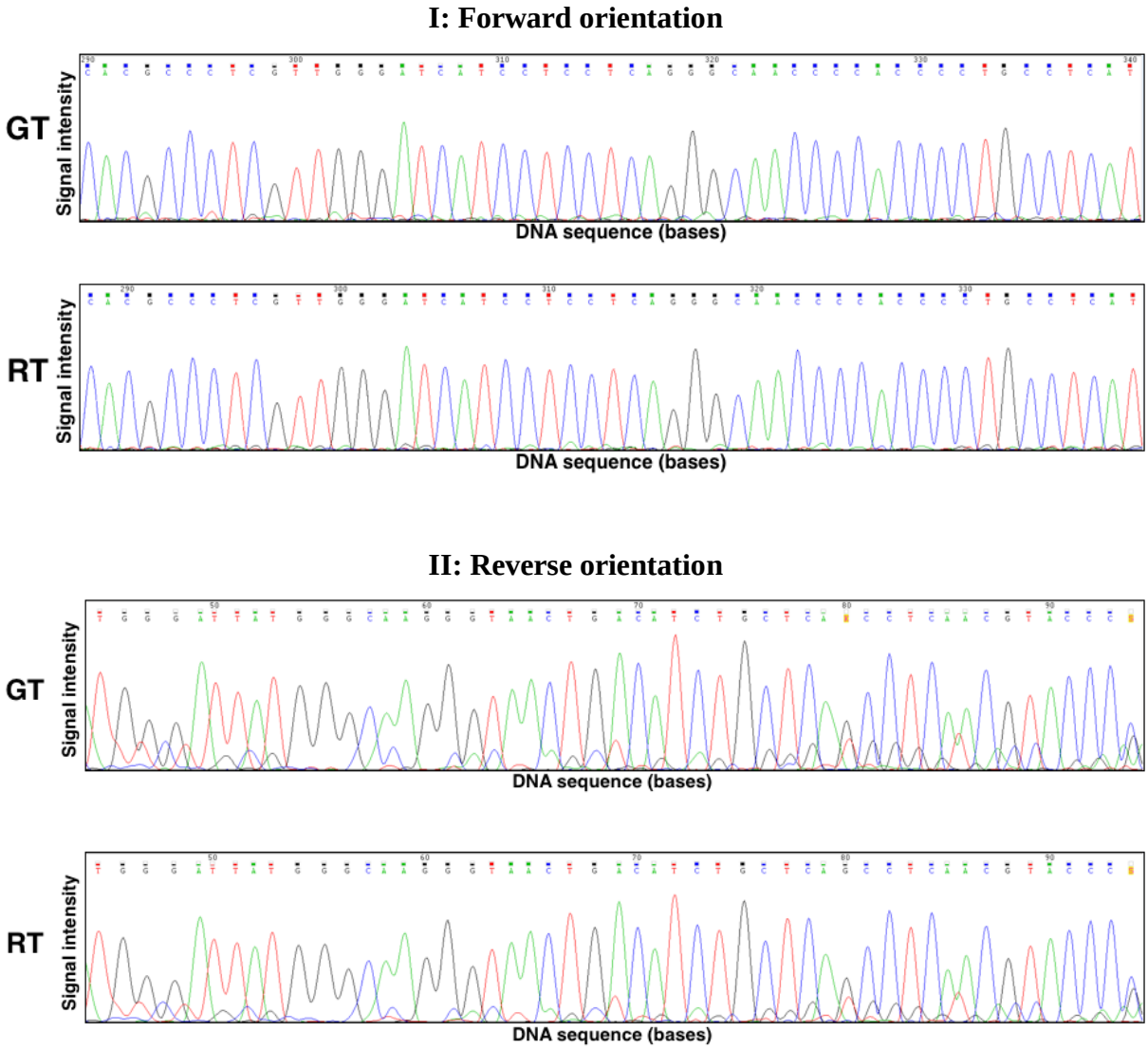
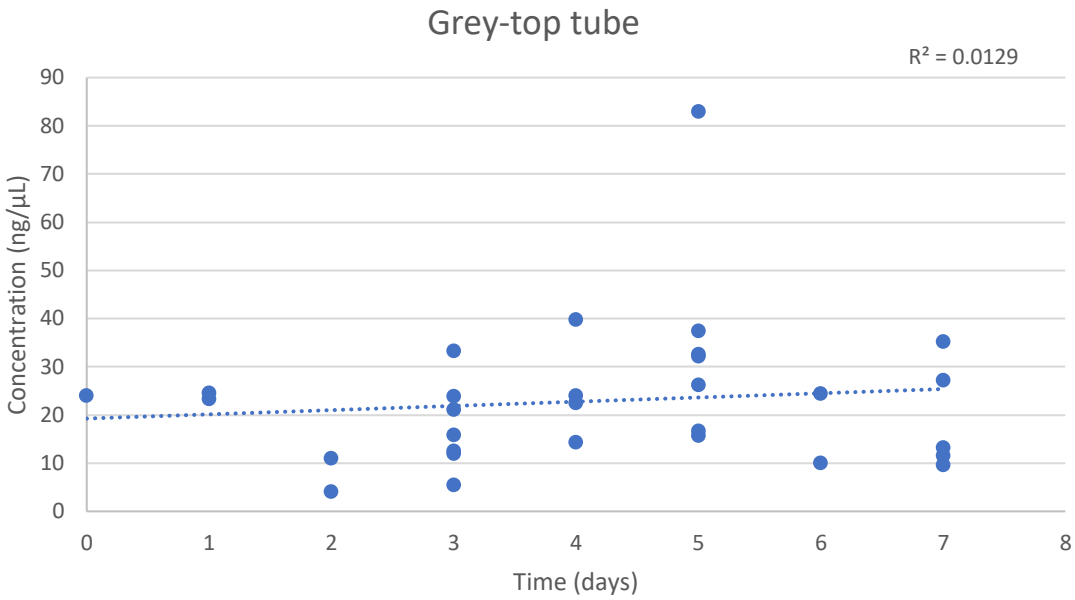


Figure S9: A portion of the DNA sequence of target I. Postmortem blood samples (4 mL) were collected into two types of blood collection tubes: grey-top (GT) and red-top (RT) tubes. The blood samples were handled in a toxicological environment (at 4˚C) and DNA was isolated approximately 16 weeks after collection using the QiaAmp DNA Investigator's Kit (Qiagen, Hilden, Germany). The targets of *CYP2D6* were amplified by PCR using primers designed in-house. The sequences of the (I) forward complement and the (II) reverse complement of target I were determined by a Sanger sequencing method that utilised the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA).

C.3. Correlation test: period between death and the collection of blood samples

I. Concentration (obtained by qPCR)

A



B

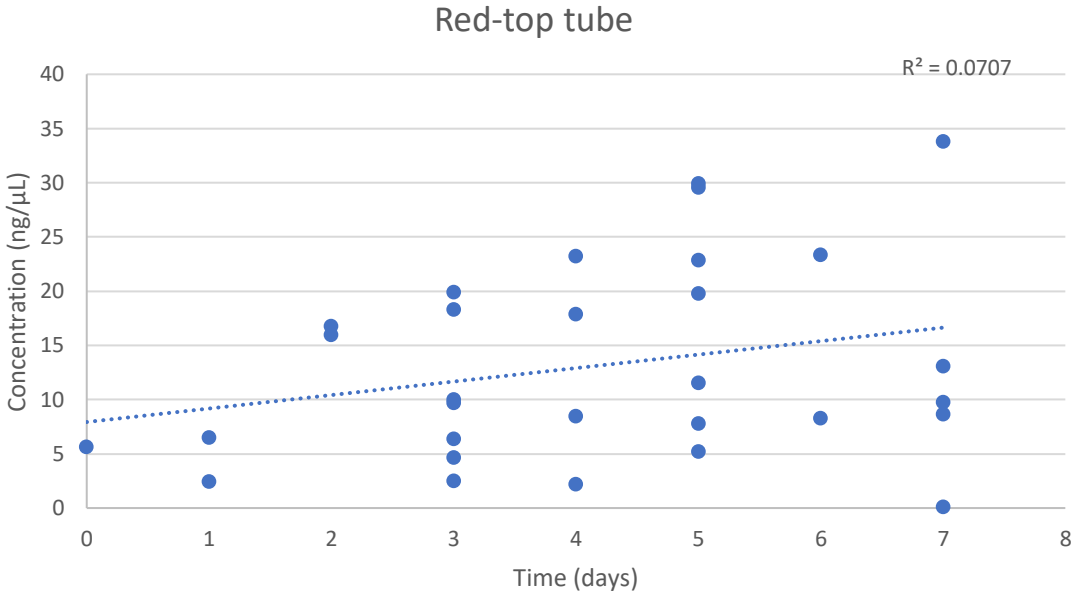
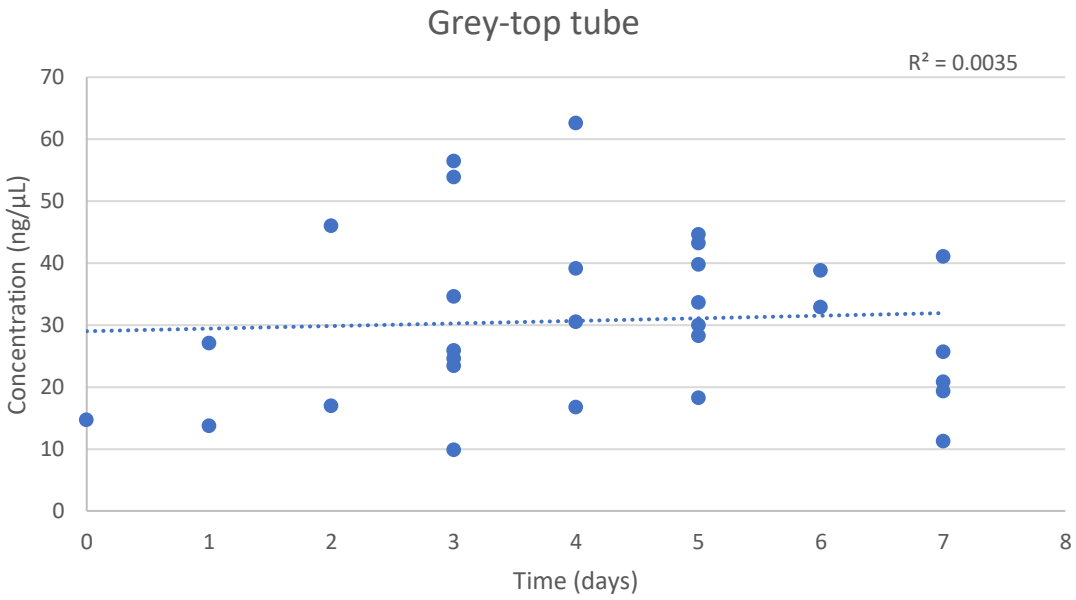


Figure S10: Correlation between DNA concentration (assessed by qPCR) and time (the period between death and the collection of blood samples). Postmortem blood samples (4 mL) were collected into two types of blood collection tubes: (**A**) grey-top and (**B**) red-top tubes. The blood samples were stored at 4˚C and DNA was isolated within 72 hours of collection from the No-Tox samples. The G-Tox and R-Tox tubes were handled in a toxicological environment (at 4˚C) and DNA was extracted approximately 16 weeks after blood collection. The QiaAmp DNA Investigator's Kit (Qiagen, Hilden, Germany) was used for DNA extraction. The DNA samples were stored at 4˚C until the Quantifiler™ Trio DNA Quantification Kit (Applied Biosystems, California, USA) was used to quantitate human DNA on a 7500 Real-Time PCR System (Applied Biosystems, California, USA).

II. Concentration (obtained by spectrophotometry)

A



B

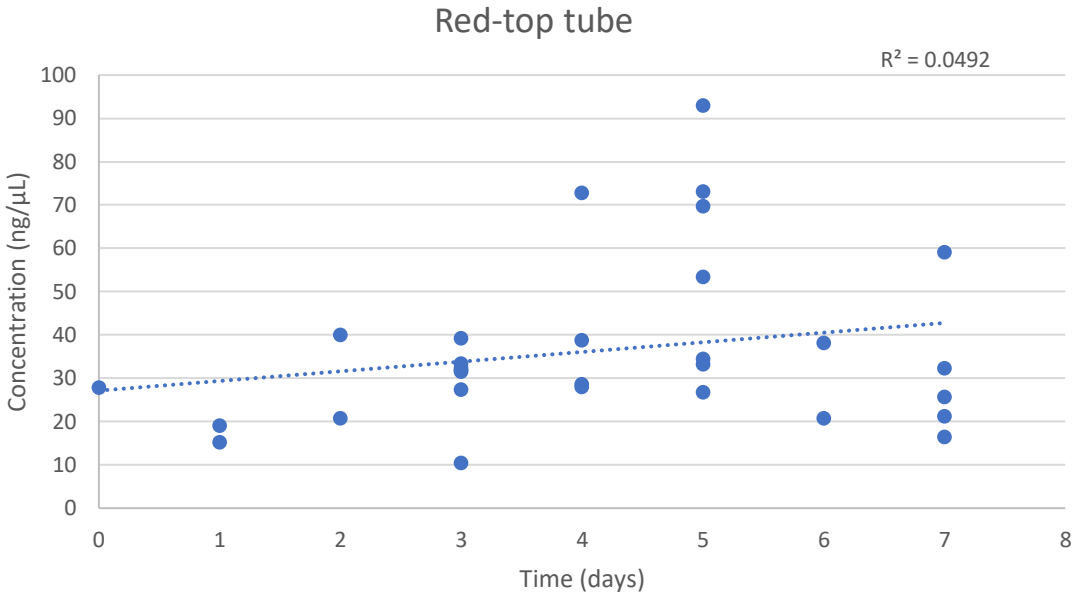
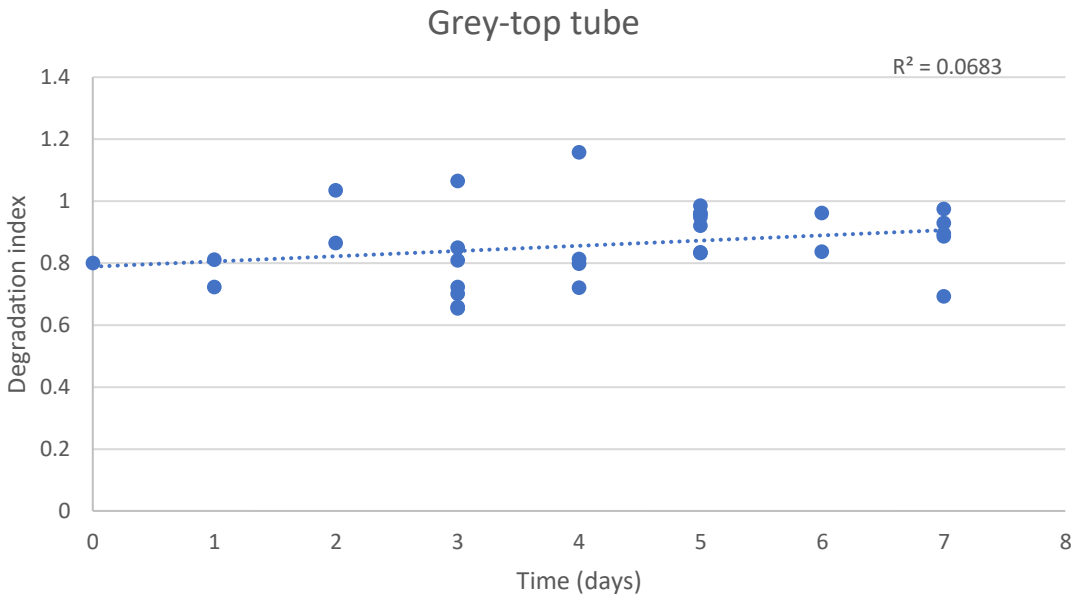


Figure S11: Correlation between DNA concentration (assessed by spectrophotometry) and time (the period between death and the collection of blood samples). Postmortem blood samples (4 mL) were collected into two types of blood collection tubes: (**A**) grey-top and (**B**) red-top tubes. The blood samples were stored at 4˚C and DNA was isolated within 72 hours of collection from the No-Tox samples. The G-Tox and R-Tox tubes were handled in a toxicological environment (at 4˚C) and DNA was extracted approximately 16 weeks after blood collection. DNA was extracted using the QiaAmp DNA Investigator's Kit (Qiagen, Hilden, Germany) and NanoDrop 2000 (Thermo Scientific, Massachusetts, USA) was used to quantitate the isolated DNA on the day of its extraction.

III. Degradation index

A



B

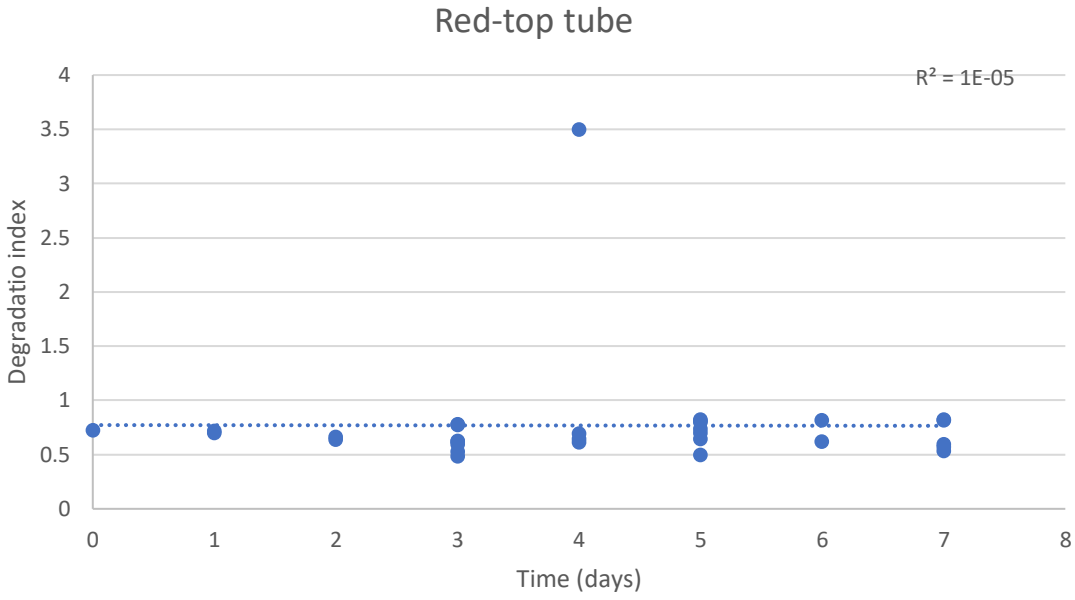
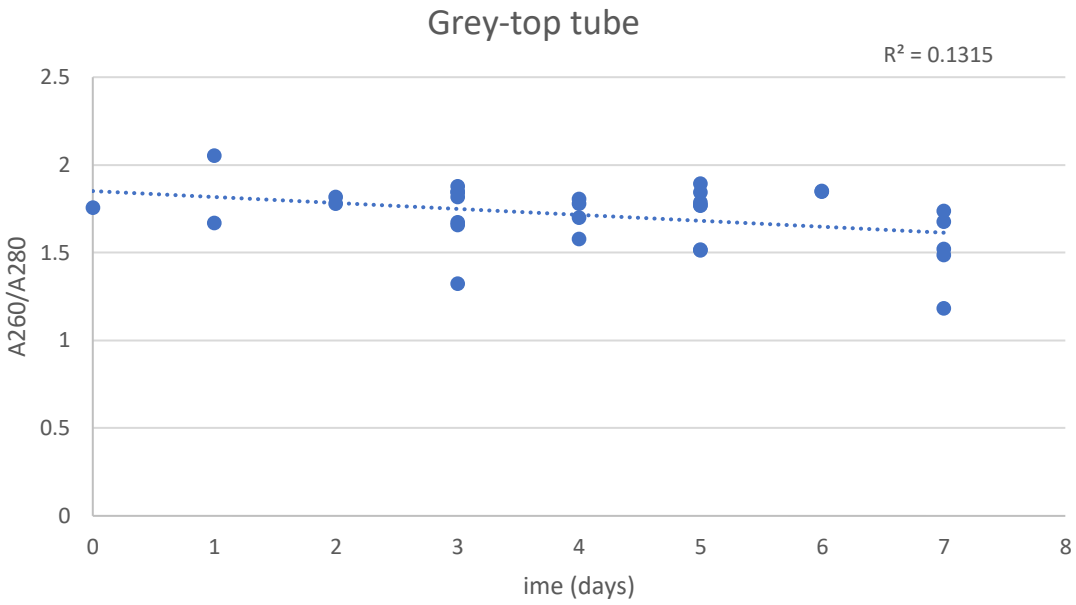


Figure S12: Correlation between degradation indices of isolated human DNA and time (the period between death and the collection of blood samples). Postmortem blood samples (4 mL) were collected into two types of blood collection tubes: (**A**) grey-top and (**B**) red-top tubes. The blood samples were stored at 4˚C and DNA was isolated within 72 hours of collection from the No-Tox samples. The G-Tox and R-Tox tubes were handled in a toxicological environment (at 4˚C) and DNA was extracted approximately 16 weeks after blood collection. The QiaAmp DNA Investigator's Kit (Qiagen, Hilden, Germany) was used for DNA extraction. The DNA samples were stored at 4˚C until the Quantifiler™ Trio DNA Quantification Kit (Applied Biosystems, California, USA) was used to determine the degradation indices of the endogenous DNA on a 7500 Real-Time PCR System (Applied Biosystems, California, USA).

IV. 260/280 Absorbance ratio

A



B

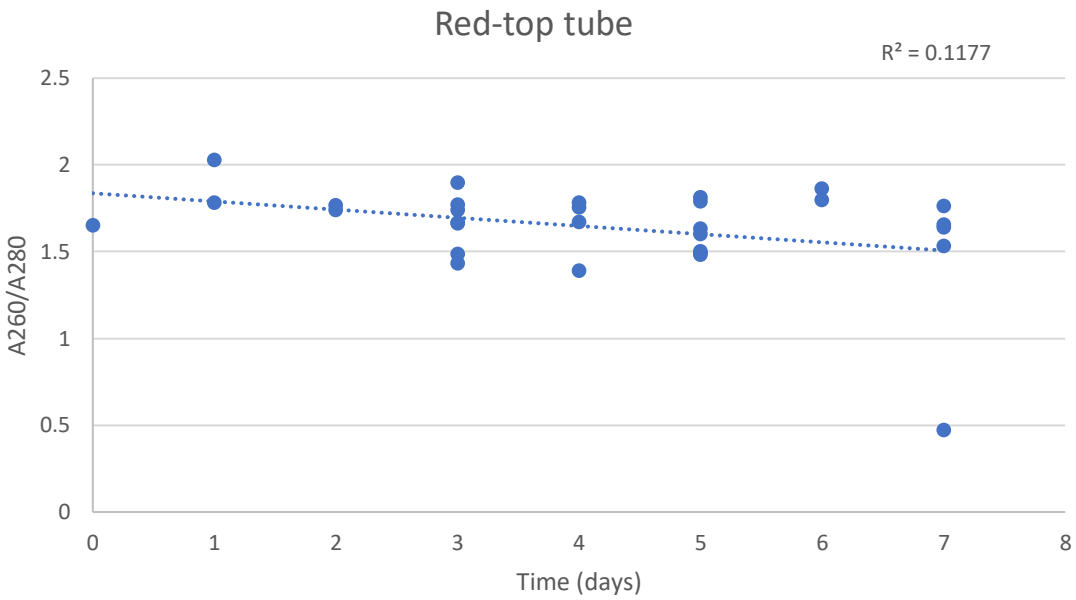
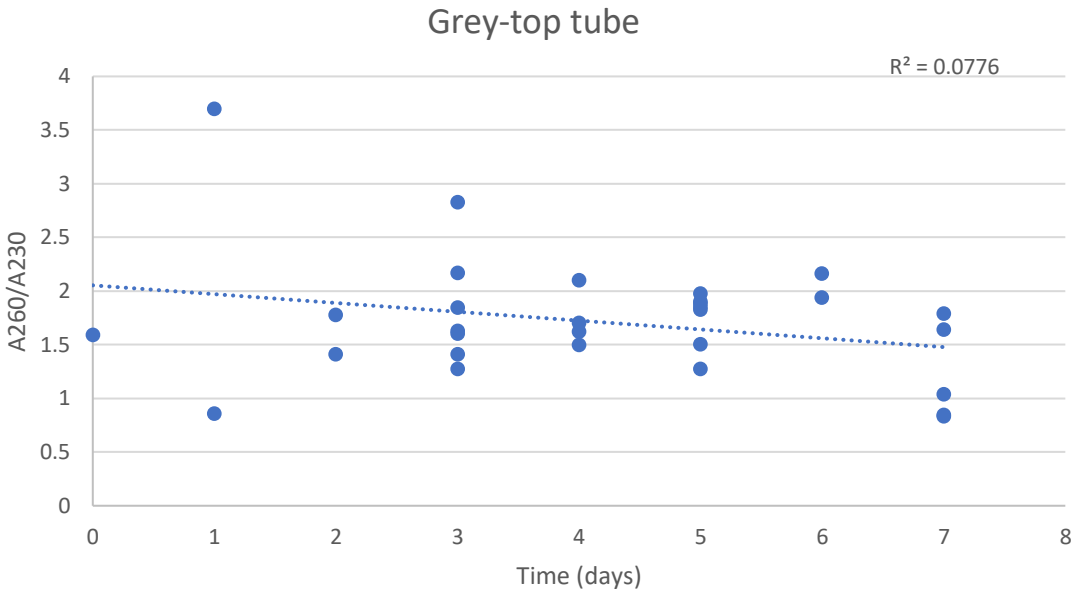


Figure S13: Correlation between absorbance ratio 260/280 and time (the period between death and the collection of blood samples). Postmortem blood samples (4 mL) were collected into two types of blood collection tubes: (**A**) grey-top and (**B**) red-top tubes. The blood samples were stored at 4˚C and DNA was isolated within 72 hours of collection from the No-Tox samples. The G-Tox and R-Tox tubes were handled in a toxicological environment (at 4˚C) and DNA was extracted approximately 16 weeks after blood collection. DNA was extracted using the QiaAmp DNA Investigator's Kit (Qiagen, Hilden, Germany) and NanoDrop 2000 (Thermo Scientific, Massachusetts, USA) was used to assess the 260/280 absorbance ratio.

V. 260/230 Absorbance ratio

A



B

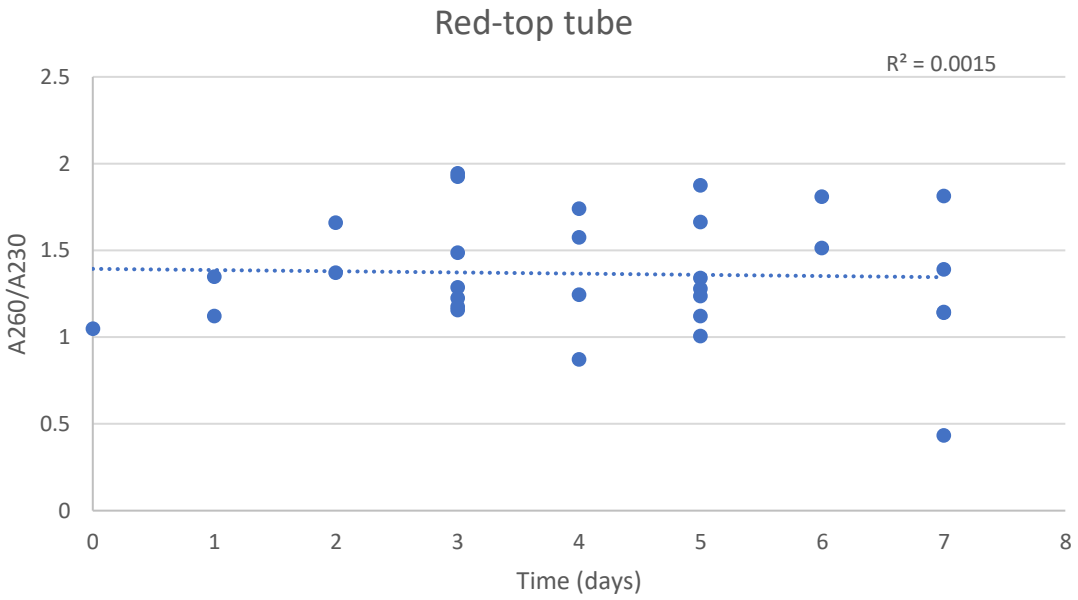
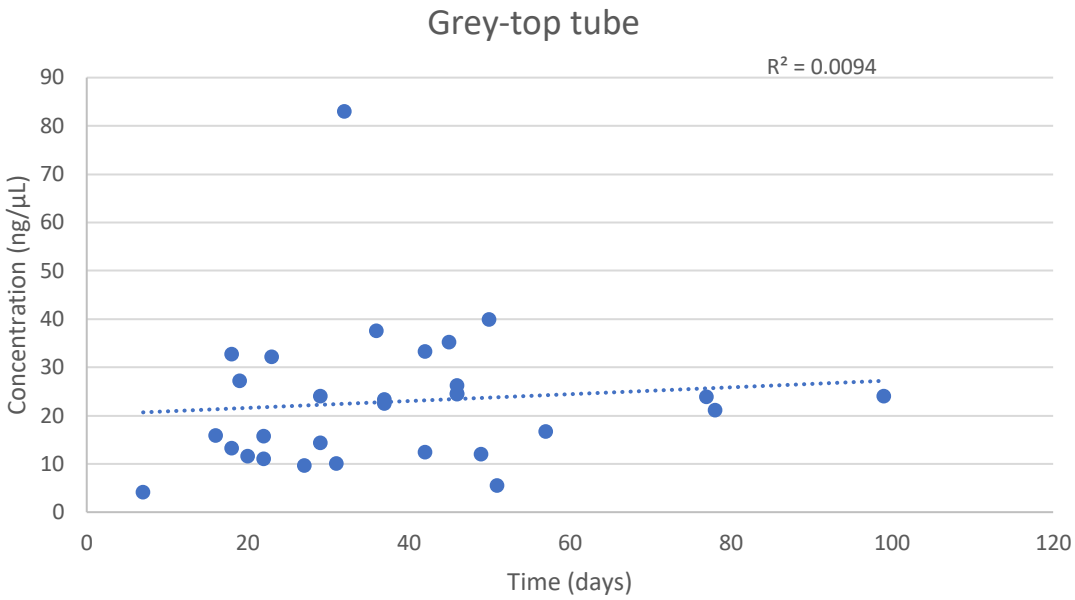


Figure S14: Correlation between absorbance ratio 260/230 and time (the period between death and the collection of blood samples). Postmortem blood samples (4 mL) were collected into two types of blood collection tubes: (**A**) grey-top and (**B**) red-top tubes. The blood samples were stored at 4˚C and DNA was isolated within 72 hours of collection from the No-Tox samples. The G-Tox and R-Tox tubes were handled in a toxicological environment (at 4˚C) and DNA was extracted approximately 16 weeks after blood collection. DNA was extracted using the QiaAmp DNA Investigator's Kit (Qiagen, Hilden, Germany) and NanoDrop 2000 (Thermo Scientific, Massachusetts, USA) was used to assess the 260/230 absorbance ratio.

C.4. Correlation test: period between death and the extraction of DNA

I. Concentration (obtained by qPCR)

A



B

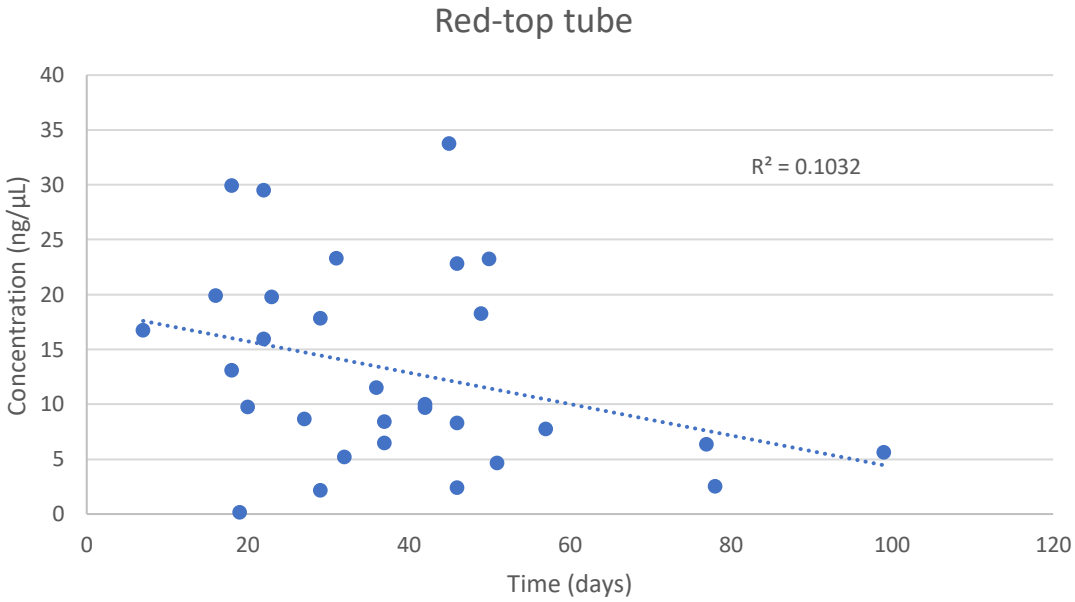
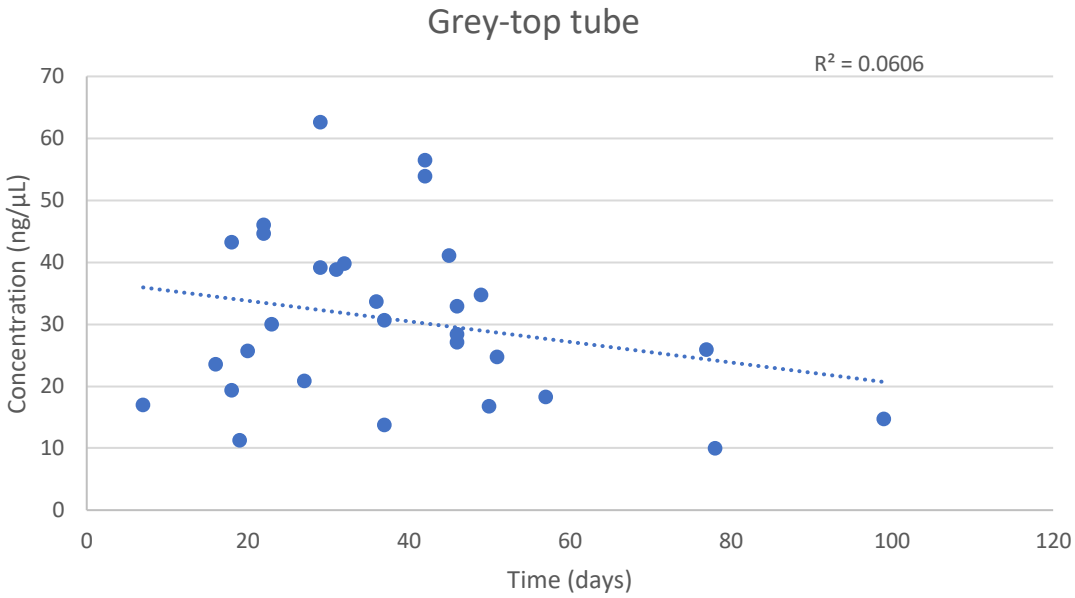


Figure 15: Correlation between DNA concentration (assessed by qPCR) and time (the period between death and the extraction of DNA). Postmortem blood samples (4 mL) were collected into two types of blood collection tubes: (**A**) grey-top and (**B**) red-top tubes. The blood samples were stored at 4˚C and DNA was isolated within 72 hours of collection from the No-Tox samples. The G-Tox and R-Tox tubes were handled in a toxicological environment (at 4˚C) and DNA was extracted approximately 16 weeks after blood collection. The QiaAmp DNA Investigator's Kit (Qiagen, Hilden, Germany) was used for DNA extraction. The DNA samples were stored at 4˚C until the Quantifiler™ Trio DNA Quantification Kit (Applied Biosystems, California, USA) was used to quantitate human DNA on a 7500 Real-Time PCR System (Applied Biosystems, California, USA).

II. Concentration (obtained by Spectrophotometry)

A



B

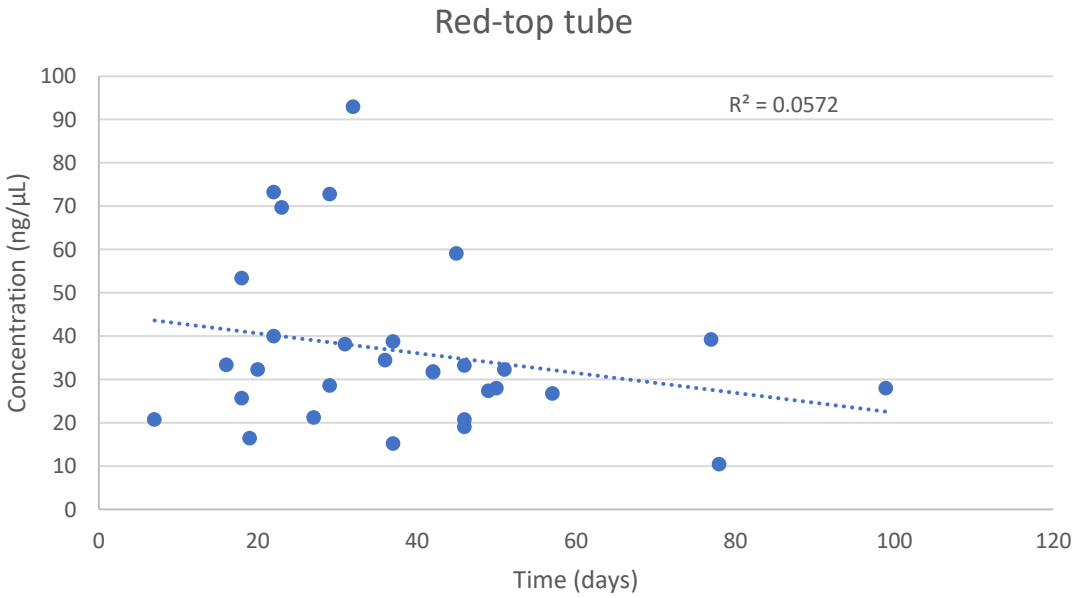
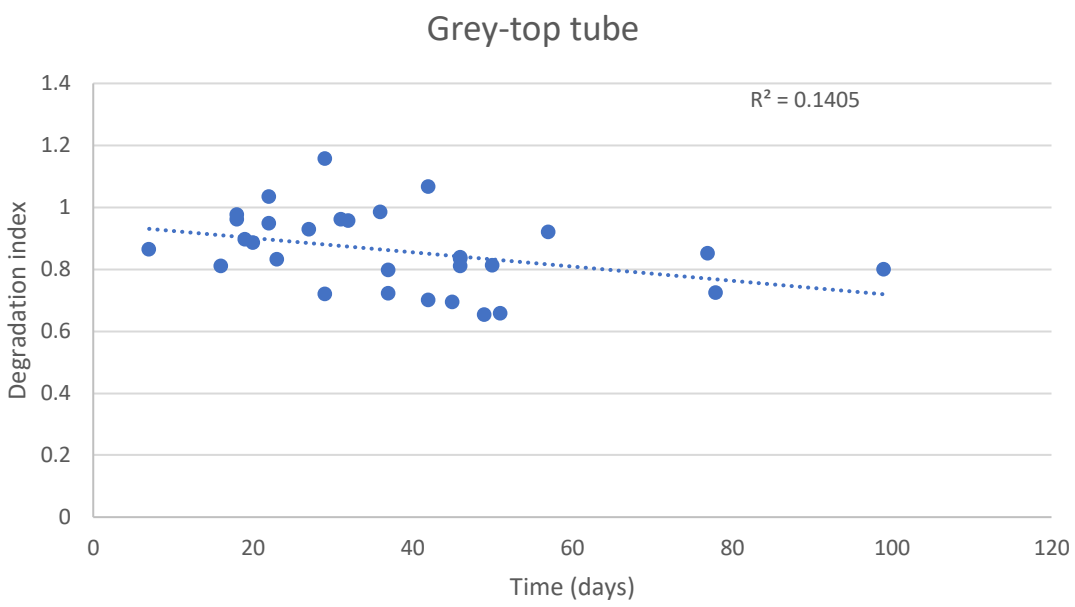


Figure S16: Correlation between DNA concentration (assessed by spectrophotometry) and time (the period between death and the extraction of DNA). Postmortem blood samples (4 mL) were collected into two types of blood collection tubes: (**A**) grey-top and (**B**) red-top tubes. The blood samples were stored at 4˚C and DNA was isolated within 72 hours of collection from the No-Tox samples. The G-Tox and R-Tox tubes were handled in a toxicological environment (at 4˚C) and DNA was extracted approximately 16 weeks after blood collection. DNA was extracted using the QiaAmp DNA Investigator's Kit (Qiagen, Hilden, Germany) and NanoDrop 2000 (Thermo Scientific, Massachusetts, USA) was used to quantitate the isolated DNA on the day of its extraction.

III. Degradation index

A



B

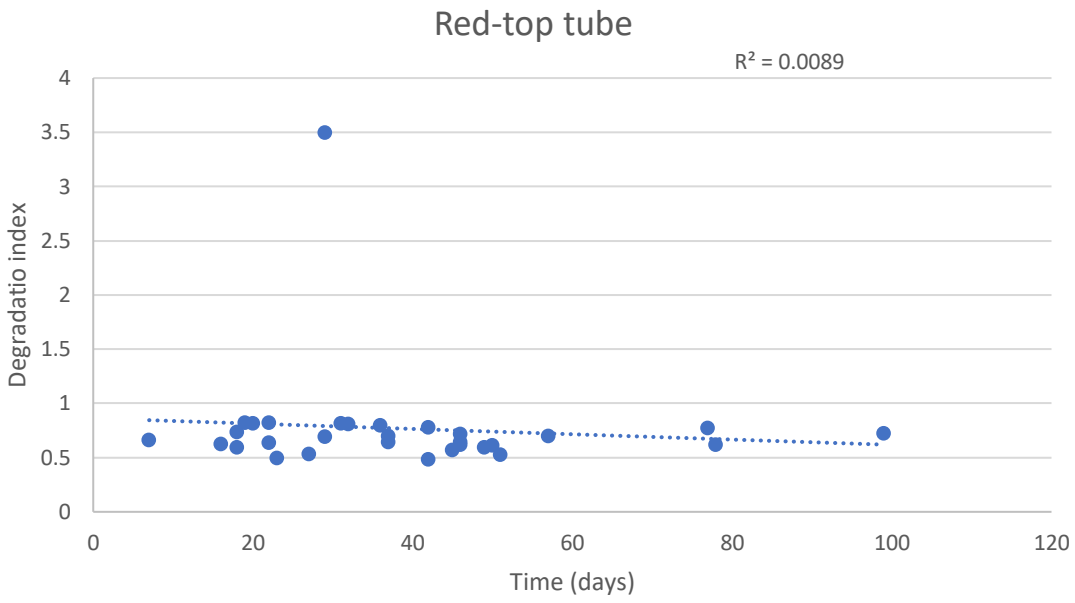
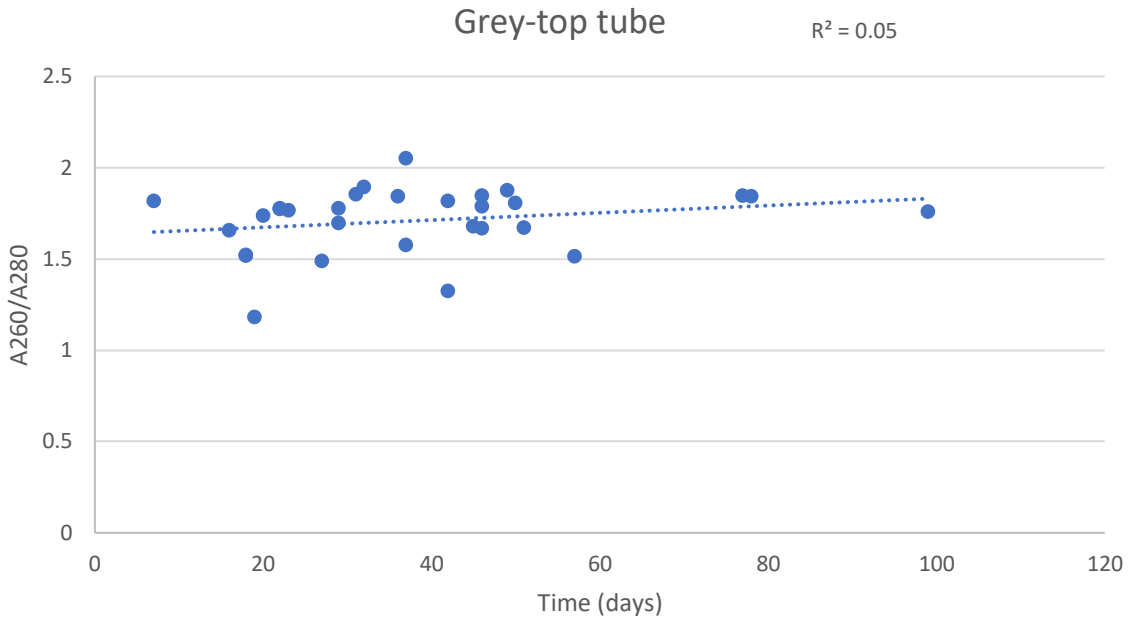


Figure 17: Correlation between degradation indices of isolated human DNA and time (the period between death and the extraction of DNA). Postmortem blood samples (4 mL) were collected into two types of blood collection tubes: (**A**) grey-top and (**B**) red-top tubes. The blood samples were stored at 4˚C and DNA was isolated within 72 hours of collection from the No-Tox samples. The G-Tox and R-Tox tubes were handled in a toxicological environment (at 4˚C) and DNA was extracted approximately 16 weeks after blood collection. The QiaAmp DNA Investigator's Kit (Qiagen, Hilden, Germany) was used for DNA extraction. The DNA samples were stored at 4˚C until the Quantifiler™ Trio DNA Quantification Kit (Applied Biosystems, California, USA) was used to determine the degradation indices of the endogenous DNA on a 7500 Real-Time PCR System (Applied Biosystems, California, USA).

IV. 260/280 Absorbance ratio

A



B

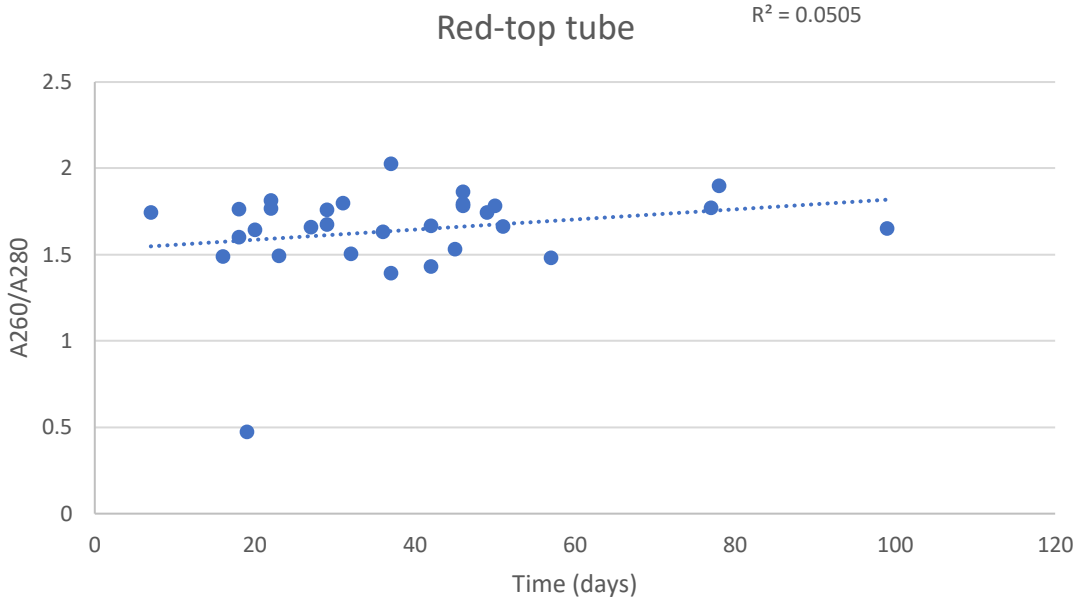
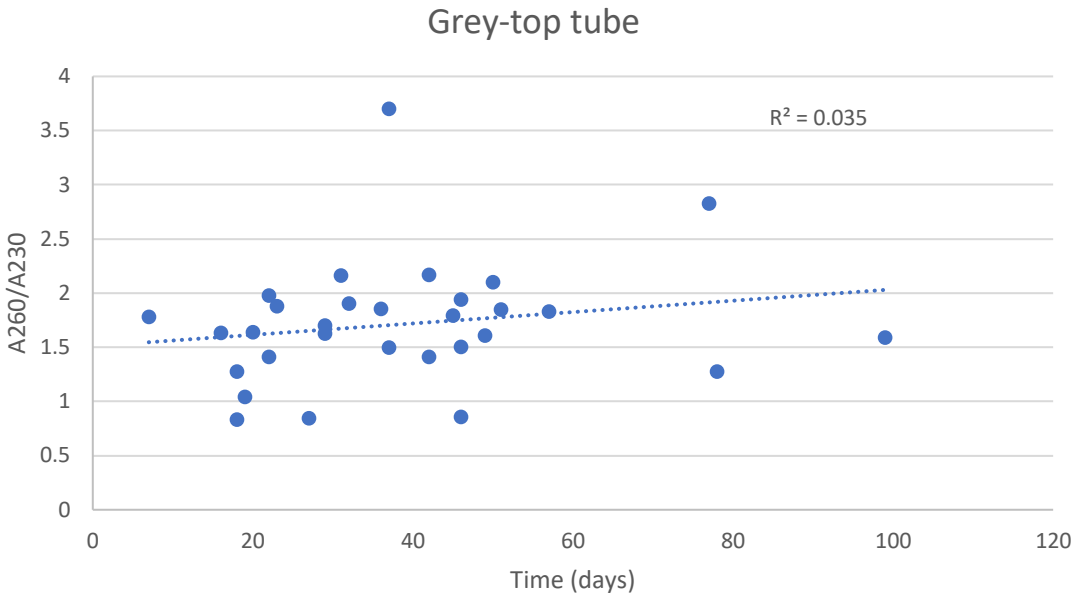


Figure 18: Correlation between absorbance ratio 260/280 and time (the period between death and the extraction of DNA). Postmortem blood samples (4 mL) were collected into two types of blood collection tubes: (**A**) grey-top and (**B**) red-top tubes. The blood samples were stored at 4˚C and DNA was isolated within 72 hours of collection from the No-Tox samples. The G-Tox and R-Tox tubes were handled in a toxicological environment (at 4˚C) and DNA was extracted approximately 16 weeks after blood collection. DNA was extracted using the QiaAmp DNA Investigator's Kit (Qiagen, Hilden, Germany) and NanoDrop 2000 (Thermo Scientific, Massachusetts, USA) was used to assess the 260/280 absorbance ratio.

V. 260/230 Absorbance ratio

A



B

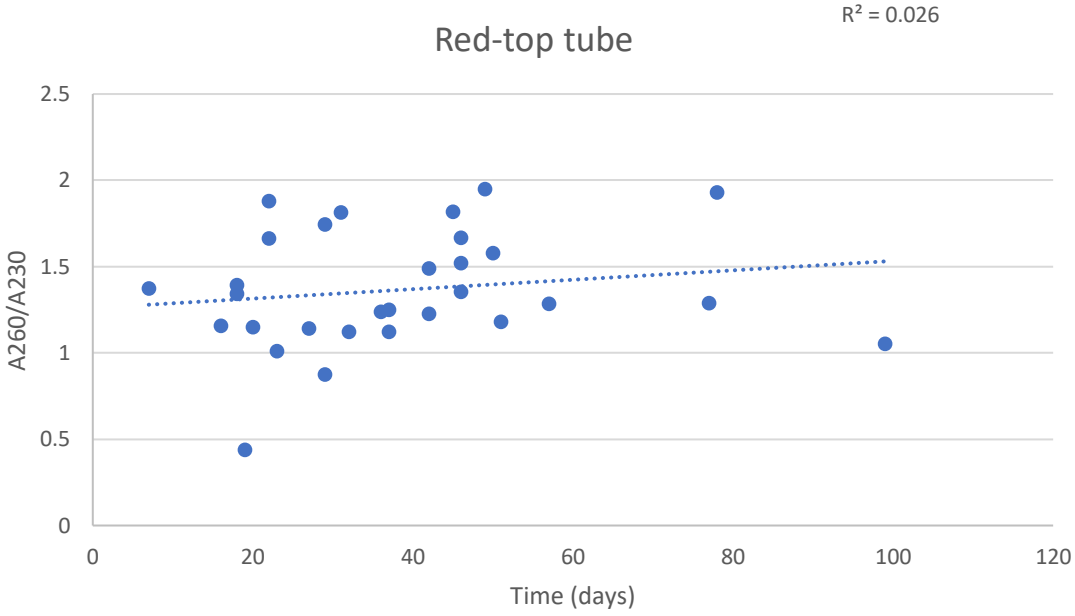


Figure 19: Correlation between absorbance ratio 260/230 and time (the period between death and the extraction of DNA). Postmortem blood samples (4 mL) were collected into two types of blood collection tubes: (**A**) grey-top and (**B**) red-top tubes. The blood samples were stored at 4˚C and DNA was isolated within 72 hours of collection from the No-Tox samples. The G-Tox and R-Tox tubes were handled in a toxicological environment (at 4˚C) and DNA was extracted approximately 16 weeks after blood collection. DNA was extracted using the QiaAmp DNA Investigator's Kit (Qiagen, Hilden, Germany) and NanoDrop 2000 (Thermo Scientific, Massachusetts, USA) was used to assess the 260/230 absorbance ratio.

APPENDIX D: STATISTICAL REPORT

1. Data

Femoral blood specimens were collected from 30 cases of deceased individuals examined at Salt River Mortuary. Blood was drawn from the femoral vein of the deceased and transferred into one 4 mL purple-top tube, two 4 mL grey-top tubes, and into two 10 mL red-top tubes. The volume of blood that was aliquoted into each of the five tubes was 4 mL. All the blood specimens were immediately transported to the University of Cape Town's Molecular Forensics' laboratory. The samples were stored at 4˚C and DNA was extracted within 72 hours of sample collection from all the samples that would not undergo toxicological analysis, identified as the "No-tox" samples: one grey-top tube (G-No-tox), one red-top tube (R-No-tox), and the purple-top tube (P-No-tox). The remaining grey-top tube and red-top tube (called G-Tox and R-Tox, respectively) remained stored at 4˚C at UCT's Forensic Toxicology Unit laboratory until downstream toxicological analysis was performed. These samples were, therefore, called "Tox" samples.

2. Objectives

In respect of DNA concentration, degradation index, and purity (A260/280 and A260/230), the objectives were:

(i) To determine if a difference exists between the purple-top tubes and the grey-top tubes that have not undergone a toxicological analysis. (P-No-Tox vs G-No-Tox)

(ii) To determine if a difference exists between the purple-top tubes and the red-top tubes that have not undergone a toxicological analysis. (P-No-Tox vs R-No-Tox)

(iii) To determine if a difference exists between the grey-top tubes and the red-top tubes that have not undergone a toxicological analysis. (G-No-Tox vs R-No-Tox)

(iv) To determine if a difference exists between the grey-top tubes and the red-top tubes that have undergone a toxicological analysis. (G-Tox vs R-Tox)

(v) To determine if a difference exists between the grey-top tubes that have undergone toxicological analysis and the grey-top tubes that have not undergone a toxicological analysis. (G-No-Tox vs G-Tox)

(vi) To determine if a difference exists between the red-top tubes that have undergone toxicological analysis and the red-top tubes that have not undergone a toxicological analysis. (R-No-Tox vs R-Tox)

3. Exploratory analysis

The samples are dependent; therefore, a matched analysis is appropriate.

3.1. Assessment of normal distribution (Shapiro Wilk test)

Overall, the data is not normally distributed (the p-values for the groups compared are provided in the log output below).

3.2. Assessment of normal distribution of difference (Shapiro Wilk Test)
None of the differences were found to be normally distributed (the p-values for the differences are provided in the log output below).

4. Hypothesis testing

The differences are not normally distributed; therefore, the data was analysed using a non-parametric test - Wilcoxon Signed Rank test, as shown in the log output provided below.

5. Log output

```
------------------------------------------------------------------------------------------------------------------------
      name:  <unnamed>
       log:  C:\Users\Administrator\Desktop\Study stats_Loyiso.log
  log type:  text
 opened on:  23 Oct 2017, 18:07:44

. import excel "C:\Users\Administrator\Desktop\Data for Stats.xlsx", sheet("qPCR Concentration") firstrow

. *Distribution of all data

. swilk PNoTox GNoTox RNoTox GTox RTox

                  Shapiro-Wilk W test for normal data

    Variable |    Obs       W           V         z       Prob>z
-------------+--------------------------------------------------
      PNoTox |     30    0.91541      2.689     2.045    0.02042
      GNoTox |     30    0.89906      3.208     2.411    0.00796
      RNoTox |     30    0.65220     11.055     4.968    0.00000
        GTox |     30    0.80237      6.282     3.800    0.00007
        RTox |     30    0.93692      2.005     1.438    0.07518

. *p<0.05 for all groups except RTox. Therefore, data is not normally distributed

. *P-No-Tox vs G-No-Tox

. signrank PNoTox= GNoTox

Wilcoxon signed-rank test

        sign |      obs   sum ranks    expected
-------------+---------------------------------
    positive |       17         263       232.5
    negative |       13         202       232.5
        zero |        0           0           0
-------------+---------------------------------
         all |       30         465         465

unadjusted variance     2363.75
adjustment for ties        0.00
adjustment for zeros       0.00
                     ----------
adjusted variance       2363.75

Ho: PNoTox = GNoTox
            z =   0.627
    Prob > |z| =   0.5304

. *p> 0.05. Therefore, there is no significant difference in the median concentrations between P-No-Tox and G-No-Tox.


. *P-No-Tox vs R-No-Tox

. signrank PNoTox= RNoTox

Wilcoxon signed-rank test

        sign |      obs   sum ranks    expected
-------------+---------------------------------
    positive |       11         137       232.5
    negative |       19         328       232.5
        zero |        0           0           0
-------------+---------------------------------
         all |       30         465         465

unadjusted variance     2363.75
adjustment for ties        0.00
adjustment for zeros       0.00
                     ----------
adjusted variance       2363.75

Ho: PNoTox = RNoTox
            z =  -1.964
    Prob > |z| =   0.0495

. *p< 0.05. Therefore, there is a significant difference in the median concentrations between P-No-Tox and G-No-Tox.
```

```
. *G-No-Tox vs R-No-Tox

. signrank GNoTox= RNoTox

Wilcoxon signed-rank test

        sign |      obs   sum ranks    expected
-------------+---------------------------------
    positive |       11         106       232.5
    negative |       19         359       232.5
        zero |        0           0           0
-------------+---------------------------------
         all |       30         465         465

unadjusted variance     2363.75
adjustment for ties        0.00
adjustment for zeros       0.00
                     ----------
adjusted variance       2363.75

Ho: GNoTox = RNoTox
              z =  -2.602
    Prob > |z| =   0.0093

. *p< 0.05. Therefore, there is a significant difference in the median concentrations between G-No-Tox and R-No-Tox

. *

. *

. *G-Tox vs R-Tox

. signrank GTox= RTox

Wilcoxon signed-rank test

        sign |      obs   sum ranks    expected
-------------+---------------------------------
    positive |       24         383       232.5
    negative |        6          82       232.5
        zero |        0           0           0
-------------+---------------------------------
         all |       30         465         465

unadjusted variance     2363.75
adjustment for ties        0.00
adjustment for zeros       0.00
                     ----------
adjusted variance       2363.75

Ho: GTox = RTox
              z =   3.096
    Prob > |z| =   0.0020

. *p< 0.05. Therefore, there is a significant difference in the median concentrations between G-Tox and R-Tox.
```

```
. *G-No-Tox vs G-Tox

. signrank GNoTox= GTox

Wilcoxon signed-rank test

        sign |      obs   sum ranks    expected
-------------+---------------------------------
    positive |       12         208       232.5
    negative |       18         257       232.5
        zero |        0           0           0
-------------+---------------------------------
         all |       30         465         465

unadjusted variance     2363.75
adjustment for ties        0.00
adjustment for zeros       0.00
                     ----------
adjusted variance       2363.75

Ho: GNoTox = GTox
             z =  -0.504
    Prob > |z| =   0.6143

. *p> 0.05. Therefore, there is no significant difference in the median concentrations between G-No-Tox and G-Tox.

. *

. *

. *R-No-Tox vs R-Tox

. signrank RNoTox= RTox

Wilcoxon signed-rank test

        sign |      obs   sum ranks    expected
-------------+---------------------------------
    positive |       25         443       232.5
    negative |        5          22       232.5
        zero |        0           0           0
-------------+---------------------------------
         all |       30         465         465

unadjusted variance     2363.75
adjustment for ties        0.00
adjustment for zeros       0.00
                     ----------
adjusted variance       2363.75

Ho: RNoTox = RTox
             z =   4.330
    Prob > |z| =   0.0000

. *p< 0.05. Therefore, there is a significant difference in the median concentrations between R-No-Tox and R-Tox.
```

```
. *Distribution of all data

. swilk PNoTox GNoTox RNoTox GTox RTox

                  Shapiro-Wilk W test for normal data

    Variable |    Obs       W           V         z       Prob>z
-------------+--------------------------------------------------
      PNoTox |     30    0.91973      2.552     1.937    0.02638
      GNoTox |     30    0.95204      1.525     0.872    0.19163
      RNoTox |     30    0.94390      1.783     1.196    0.11584
        GTox |     30    0.96615      1.076     0.151    0.43987
        RTox |     30    0.85827      4.505     3.112    0.00093

. *p<0.05 for PNoTox and RTox. Therefore, data (on Nanodrop concentrations) is not normally distributed.

. *P-No-Tox vs G-No-Tox

. signrank PNoTox= GNoTox

Wilcoxon signed-rank test

        sign |      obs   sum ranks    expected
-------------+---------------------------------
    positive |       19         309       232.5
    negative |       11         156       232.5
        zero |        0           0           0
-------------+---------------------------------
         all |       30         465         465

unadjusted variance     2363.75
adjustment for ties        0.00
adjustment for zeros       0.00
                     ----------
adjusted variance       2363.75

Ho: PNoTox = GNoTox
             z =   1.573
    Prob > |z| =   0.1156

. *p> 0.05. Therefore, there is no significant difference in the median concentrations between P-No-Tox and G-No-Tox.
```

```
. *P-No-Tox vs R-No-Tox

. signrank PNoTox= RNoTox

Wilcoxon signed-rank test

        sign |      obs   sum ranks    expected
-------------+---------------------------------
    positive |        9          80       232.5
    negative |       21         385       232.5
        zero |        0           0           0
-------------+---------------------------------
         all |       30         465         465

unadjusted variance     2363.75
adjustment for ties        0.00
adjustment for zeros       0.00
                     ----------
adjusted variance       2363.75

Ho: PNoTox = RNoTox
              z =  -3.137
    Prob > |z| =   0.0017

. *p< 0.05. Therefore, there is a significant difference in the median concentrations between P-No-Tox and R-No-Tox.

. *

. *

. *G-No-Tox vs R-No-Tox

. signrank GNoTox= RNoTox

Wilcoxon signed-rank test

        sign |      obs   sum ranks    expected
-------------+---------------------------------
    positive |        4        32.5       232.5
    negative |       26       432.5       232.5
        zero |        0           0           0
-------------+---------------------------------
         all |       30         465         465

unadjusted variance     2363.75
adjustment for ties       -0.25
adjustment for zeros       0.00
                     ----------
adjusted variance       2363.50

Ho: GNoTox = RNoTox
              z =  -4.114
    Prob > |z| =   0.0000

. *p< 0.05. Therefore, there is a significant difference in the median concentrations between G-No-Tox and R-No-Tox
```

```
. *G-Tox vs R-Tox

. signrank GTox= RTox

Wilcoxon signed-rank test

        sign |      obs   sum ranks    expected
-------------+---------------------------------
    positive |        8         133       232.5
    negative |       22         332       232.5
        zero |        0           0           0
-------------+---------------------------------
         all |       30         465         465

unadjusted variance     2363.75
adjustment for ties        0.00
adjustment for zeros       0.00
                     ----------
adjusted variance       2363.75

Ho: GTox = RTox
             z =  -2.047
    Prob > |z| =   0.0407

. *p< 0.05. Therefore, there is a significant difference in the median concentrations between G-Tox and R-Tox.

. *

. *

. *G-No-Tox vs G-Tox

. signrank GNoTox= GTox

Wilcoxon signed-rank test

        sign |      obs   sum ranks    expected
-------------+---------------------------------
    positive |       16         262       232.5
    negative |       14         203       232.5
        zero |        0           0           0
-------------+---------------------------------
         all |       30         465         465

unadjusted variance     2363.75
adjustment for ties        0.00
adjustment for zeros       0.00
                     ----------
adjusted variance       2363.75

Ho: GNoTox = GTox
             z =   0.607
    Prob > |z| =   0.5440

. *p> 0.05. Therefore, there is no significant difference in the median concentrations between G-No-Tox and G-Tox.

. *R-No-Tox vs R-Tox

. signrank RNoTox= RTox

Wilcoxon signed-rank test

        sign |      obs   sum ranks    expected
-------------+---------------------------------
    positive |       19         338       232.5
    negative |       11         127       232.5
        zero |        0           0           0
-------------+---------------------------------
         all |       30         465         465

unadjusted variance     2363.75
adjustment for ties        0.00
adjustment for zeros       0.00
                     ----------
adjusted variance       2363.75

Ho: RNoTox = RTox
             z =   2.170
    Prob > |z| =   0.0300

. *p< 0.05. Therefore, there is a significant difference in the median concentrations between R-No-Tox and R-Tox.
```

```
. *P-No-Tox vs R-No-Tox

. signrank PNoTox= RNoTox

Wilcoxon signed-rank test

        sign |      obs   sum ranks    expected
-------------+---------------------------------
    positive |       19         331       232.5
    negative |       11         134       232.5
        zero |        0           0           0
-------------+---------------------------------
         all |       30         465         465

unadjusted variance     2363.75
adjustment for ties        0.00
adjustment for zeros       0.00
                     ----------
adjusted variance       2363.75

Ho: PNoTox = RNoTox
              z =   2.026
    Prob > |z| =   0.0428

. *p< 0.05. Therefore, there is a significant difference in the median degradation index between P-No-Tox and G-No-Tox.

. *

. *

. *G-No-Tox vs R-No-Tox

. signrank GNoTox= RNoTox

Wilcoxon signed-rank test

        sign |      obs   sum ranks    expected
-------------+---------------------------------
    positive |       21         359       232.5
    negative |        9         106       232.5
        zero |        0           0           0
-------------+---------------------------------
         all |       30         465         465

unadjusted variance     2363.75
adjustment for ties        0.00
adjustment for zeros       0.00
                     ----------
adjusted variance       2363.75

Ho: GNoTox = RNoTox
              z =   2.602
    Prob > |z| =   0.0093

. *p< 0.05. Therefore, there is a significant difference in the median degradation index between G-No-Tox and R-No-Tox
```

```
. *DEGRADATION INDEX

. import excel "C:\Users\Administrator\Desktop\Data for Stats.xlsx", sheet("Degradation index") firstrow clear

. *Distribution of all data

. swilk PNoTox GNoTox RNoTox GTox RTox

                    Shapiro-Wilk W test for normal data

    Variable |    Obs       W           V         z       Prob>z
-------------+--------------------------------------------------
      PNoTox |     30    0.61657     12.187     5.170    0.00000
      GNoTox |     30    0.91426      2.725     2.073    0.01909
      RNoTox |     30    0.96602      1.080     0.159    0.43666
        GTox |     30    0.97470      0.804    -0.451    0.67396
        RTox |     30    0.34446     20.836     6.279    0.00000

. *p<0.05 for all groups except RNoTox and GTox. Therefore, data is not normally distributed

. *P-No-Tox vs G-No-Tox

. signrank PNoTox= GNoTox

Wilcoxon signed-rank test

        sign |      obs   sum ranks    expected
-------------+---------------------------------
    positive |       14         220       232.5
    negative |       16         245       232.5
        zero |        0           0           0
-------------+---------------------------------
         all |       30         465         465

unadjusted variance     2363.75
adjustment for ties        0.00
adjustment for zeros       0.00
                     ----------
adjusted variance       2363.75

Ho: PNoTox = GNoTox
             z =  -0.257
    Prob > |z| =   0.7971

. *p> 0.05. Therefore, there is no significant difference in the median degradation index between P-No-Tox and G-No-Tox.
```

```
. *G-Tox vs R-Tox

. signrank GTox= RTox

Wilcoxon signed-rank test

        sign |      obs   sum ranks    expected
-------------+---------------------------------
    positive |       29         435       232.5
    negative |        1          30       232.5
        zero |        0           0           0
-------------+---------------------------------
         all |       30         465         465

unadjusted variance     2363.75
adjustment for ties        0.00
adjustment for zeros       0.00
                     ----------
adjusted variance       2363.75

Ho: GTox = RTox
            z =   4.165
    Prob > |z| =   0.0000

. *p< 0.05. Therefore, there is a significant difference in the median degradation index between G-Tox and R-Tox.

. *

. *

. *G-No-Tox vs G-Tox

. signrank GNoTox= GTox

Wilcoxon signed-rank test

        sign |      obs   sum ranks    expected
-------------+---------------------------------
    positive |       14         243       232.5
    negative |       16         222       232.5
        zero |        0           0           0
-------------+---------------------------------
         all |       30         465         465

unadjusted variance     2363.75
adjustment for ties        0.00
adjustment for zeros       0.00
                     ----------
adjusted variance       2363.75

Ho: GNoTox = GTox
            z =   0.216
    Prob > |z| =   0.8290

. *p> 0.05. Therefore, there is no significant difference in the median degradation index between G-No-Tox and G-Tox.
```

```
. *R-No-Tox vs R-Tox

. signrank RNoTox= RTox

Wilcoxon signed-rank test

        sign |      obs   sum ranks    expected
-------------+---------------------------------
    positive |       25         422       232.5
    negative |        5          43       232.5
        zero |        0           0           0
-------------+---------------------------------
         all |       30         465         465

unadjusted variance     2363.75
adjustment for ties        0.00
adjustment for zeros       0.00
                     ----------
adjusted variance       2363.75

Ho: RNoTox = RTox
             z =   3.898
    Prob > |z| =   0.0001

. *p< 0.05. Therefore, there is a significant difference in the median degradation index between R-No-Tox and R-Tox.

. *

. *

. *Absrobance ratio (260/280)

. import excel "C:\Users\Administrator\Desktop\Data for Stats.xlsx", sheet("260-280 Absorbance ratio") firstrow clear

. *Distribution of all data

. swilk PNoTox GNoTox RNoTox GTox RTox

                  Shapiro-Wilk W test for normal data

    Variable |    Obs       W           V         z       Prob>z
-------------+-------------------------------------------------
      PNoTox |     30    0.73369      8.465     4.416    0.00001
      GNoTox |     30    0.89601      3.305     2.472    0.00672
      RNoTox |     30    0.96923      0.978    -0.046    0.51837
        GTox |     30    0.90871      2.902     2.203    0.01381
        RTox |     30    0.71338      9.110     4.568    0.00000

. *p<0.05 for all groups except RNoTox. Therefore, data is not normally distributed
```

```
. *P-No-Tox vs G-No-Tox

. signrank PNoTox= GNoTox

Wilcoxon signed-rank test

        sign |      obs   sum ranks    expected
-------------+---------------------------------
    positive |       13       167.5       232.5
    negative |       17       297.5       232.5
        zero |        0           0           0
-------------+---------------------------------
         all |       30         465         465

unadjusted variance     2363.75
adjustment for ties       -1.25
adjustment for zeros       0.00
                     ----------
adjusted variance       2362.50

Ho: PNoTox = GNoTox
             z =  -1.337
    Prob > |z| =   0.1811

. *p> 0.05. Therefore, there is no significant difference in the median A260/280 between P-No-Tox and G-No-Tox.

. *

. *

. *P-No-Tox vs R-No-Tox

. signrank PNoTox= RNoTox

Wilcoxon signed-rank test

        sign |      obs   sum ranks    expected
-------------+---------------------------------
    positive |       13       185.5       232.5
    negative |       17       279.5       232.5
        zero |        0           0           0
-------------+---------------------------------
         all |       30         465         465

unadjusted variance     2363.75
adjustment for ties       -2.13
adjustment for zeros       0.00
                     ----------
adjusted variance       2361.63

Ho: PNoTox = RNoTox
             z =  -0.967
    Prob > |z| =   0.3335

. *p> 0.05. Therefore, there is no significant difference in the median A260/280 between P-No-Tox and R-No-Tox.
```

```
. *G-No-Tox vs R-No-Tox

. signrank GNoTox= RNoTox

Wilcoxon signed-rank test

        sign |      obs   sum ranks    expected
-------------+---------------------------------
    positive |       16         266         232
    negative |       13         198         232
        zero |        1           1           1
-------------+---------------------------------
         all |       30         465         465

unadjusted variance     2363.75
adjustment for ties       -1.13
adjustment for zeros      -0.25
                     ----------
adjusted variance       2362.38

Ho: GNoTox = RNoTox
             z =   0.700
    Prob > |z| =   0.4842

. *p> 0.05. Therefore, there is no significant difference in the median A260/280 between G-No-Tox and R-No-Tox.

. *

. *

. *G-Tox vs R-Tox

. signrank GTox= RTox

Wilcoxon signed-rank test

        sign |      obs   sum ranks    expected
-------------+---------------------------------
    positive |       20       330.5       232.5
    negative |       10       134.5       232.5
        zero |        0           0           0
-------------+---------------------------------
         all |       30         465         465

unadjusted variance     2363.75
adjustment for ties       -0.63
adjustment for zeros       0.00
                     ----------
adjusted variance       2363.13

Ho: GTox = RTox
             z =   2.016
    Prob > |z| =   0.0438

. *p< 0.05. Therefore, there is a significant difference in the median A260/280 between G-Tox and R-Tox.
```

```
. *G-No-Tox vs G-Tox

. signrank GNoTox= GTox

Wilcoxon signed-rank test

        sign |      obs   sum ranks    expected
-------------+---------------------------------
    positive |       24       413.5         232
    negative |        5        50.5         232
        zero |        1           1           1
-------------+---------------------------------
         all |       30         465         465

unadjusted variance     2363.75
adjustment for ties       -0.38
adjustment for zeros      -0.25
                     ----------
adjusted variance       2363.13

Ho: GNoTox = GTox
            z =   3.734
    Prob > |z| =   0.0002

. *p< 0.05. Therefore, there is a significant difference in the median A260/280 between G-No-Tox and G-Tox.

. *

. *

. *R-No-Tox vs R-Tox

. signrank RNoTox= RTox

Wilcoxon signed-rank test

        sign |      obs   sum ranks    expected
-------------+---------------------------------
    positive |       26         430         232
    negative |        3          34         232
        zero |        1           1           1
-------------+---------------------------------
         all |       30         465         465

unadjusted variance     2363.75
adjustment for ties       -0.38
adjustment for zeros      -0.25
                     ----------
adjusted variance       2363.13

Ho: RNoTox = RTox
            z =   4.073
    Prob > |z| =   0.0000

. *p< 0.05. Therefore, there is a significant difference in the median A260/280 between R-No-Tox and R-Tox.
```

```
. *Absrobance ratio (260/230)

. import excel "C:\Users\Administrator\Desktop\Data for Stats.xlsx", sheet("260-230 Absorbance ratio") firstrow clear

. *Distribution of all data

. swilk PNoTox GNoTox RNoTox GTox RTox

                    Shapiro-Wilk W test for normal data

    Variable |    Obs       W           V         z       Prob>z
-------------+--------------------------------------------------
      PNoTox |     30    0.95758      1.348     0.618    0.26831
      GNoTox |     30    0.96477      1.120     0.234    0.40742
      RNoTox |     30    0.96730      1.039     0.080    0.46809
       GTox |     30    0.88153      3.766     2.742    0.00306
       RTox |     30    0.95692      1.369     0.650    0.25790

. *p<0.05 for G-Tox. Therefore, data is not normally distributed.

. *

. *

. *P-No-Tox vs G-No-Tox

. signrank PNoTox= GNoTox

Wilcoxon signed-rank test

        sign |      obs   sum ranks    expected
-------------+---------------------------------
    positive |       13         179       232.5
    negative |       17         286       232.5
        zero |        0           0           0
-------------+---------------------------------
         all |       30         465         465

unadjusted variance     2363.75
adjustment for ties        0.00
adjustment for zeros       0.00
                     ----------
adjusted variance       2363.75

Ho: PNoTox = GNoTox
              z =  -1.100
    Prob > |z| =   0.2712

. *p> 0.05. Therefore, there is no significant difference in the median A260/230 between P-No-Tox and G-No-Tox.
```

```
. *G-Tox vs R-Tox

. signrank GTox= RTox

Wilcoxon signed-rank test

        sign |      obs   sum ranks    expected
-------------+---------------------------------
    positive |       19         361       232.5
    negative |       11         104       232.5
        zero |        0           0           0
-------------+---------------------------------
         all |       30         465         465

unadjusted variance     2363.75
adjustment for ties        0.00
adjustment for zeros       0.00
                     ----------
adjusted variance       2363.75

Ho: GTox = RTox
             z =   2.643
    Prob > |z| =   0.0082

. *p< 0.05. Therefore, there is a significant difference in the median A260/230 between G-Tox and R-Tox.

. *

. *

. *G-No-Tox vs G-Tox

. signrank GNoTox= GTox

Wilcoxon signed-rank test

        sign |      obs   sum ranks    expected
-------------+---------------------------------
    positive |       10         164       232.5
    negative |       20         301       232.5
        zero |        0           0           0
-------------+---------------------------------
         all |       30         465         465

unadjusted variance     2363.75
adjustment for ties        0.00
adjustment for zeros       0.00
                     ----------
adjusted variance       2363.75

Ho: GNoTox = GTox
             z =  -1.409
    Prob > |z| =   0.1589

. *p> 0.05. Therefore, there is no significant difference in the median A260/230 between G-No-Tox and G-Tox.
```

```
. *P-No-Tox vs R-No-Tox

. signrank PNoTox= RNoTox

Wilcoxon signed-rank test

        sign |      obs   sum ranks    expected
-------------+---------------------------------
    positive |       15       264.5       232.5
    negative |       15       200.5       232.5
        zero |        0           0           0
-------------+---------------------------------
         all |       30         465         465

unadjusted variance     2363.75
adjustment for ties       -0.25
adjustment for zeros       0.00
                     ----------
adjusted variance       2363.50

Ho: PNoTox = RNoTox
             z =   0.658
    Prob > |z| =   0.5104

. *p> 0.05. Therefore, there is a significant difference in the median A260/230 between P-No-Tox and R-No-Tox.

. *

. *

. *G-No-Tox vs R-No-Tox

. signrank GNoTox= RNoTox

Wilcoxon signed-rank test

        sign |      obs   sum ranks    expected
-------------+---------------------------------
    positive |       17         331       232.5
    negative |       13         134       232.5
        zero |        0           0           0
-------------+---------------------------------
         all |       30         465         465

unadjusted variance     2363.75
adjustment for ties       -0.13
adjustment for zeros       0.00
                     ----------
adjusted variance       2363.63

Ho: GNoTox = RNoTox
             z =   2.026
    Prob > |z| =   0.0428

. *p< 0.05. Therefore, there is a significant difference in the median A260/230 between G-No-Tox and R-No-Tox.

. *R-No-Tox vs R-Tox

. signrank RNoTox= RTox

Wilcoxon signed-rank test

        sign |      obs   sum ranks    expected
-------------+---------------------------------
    positive |       14       208.5       232.5
    negative |       16       256.5       232.5
        zero |        0           0           0
-------------+---------------------------------
         all |       30         465         465

unadjusted variance     2363.75
adjustment for ties       -0.25
adjustment for zeros       0.00
                     ----------
adjusted variance       2363.50

Ho: RNoTox = RTox
             z =  -0.494
    Prob > |z| =   0.6215

. *p> 0.05. Therefore, there is no significant difference in the median A260/230 between R-No-Tox and R-Tox.

. save "C:\Users\Administrator\Desktop\Study stats_Loyiso.dta"
file C:\Users\Administrator\Desktop\Study stats_Loyiso.dta saved

. exit, clear
```