Assessment of the suitability of blood samples collected for toxicological analysis for subsequent genetic analysis: A follow-up study one year later

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LIST OF ABBREVIATIONS AND SYMBOLS

%:	Percentage
^{o}C:	Degrees Celsius
$A_{260/230}$:	Ratio of absorbance at 260 to absorbance at 230 nanometres
$A_{260/280}$:	Ratio of absorbance at 260 to absorbance at 280 nanometres
ADR:	Adverse drug reactions
bp:	Base pairs
$C_2K_2O_4$:	Potassium oxalate
CoD:	Cause of death
Ct:	Cycle threshold
CT:	Cape Town
CYP:	Cytochrome P450
DI:	Degradation index
DME:	Drug metabolising enzymes
DNA:	Deoxyribonucleic acid
dNTPs:	Deoxyribonucleotide triphosphates
DoH:	Department of Health
EDTA:	Ethylene diamine tetra-acetic acid
EM:	Extensive metaboliser
EPG:	Electropherogram
FCL:	Forensic Chemistry Laboratory
Fe:	Iron
FFPE:	Formalin fixed paraffin-embedded
FPS:	Forensic Pathology Service
GT:	Grey-top sample
HREC:	Human Research Ethics Committee
i.e.:	In other words
IM:	Intermediate metaboliser
IPC:	Internal positive control

kb:	Kilo bases
mA:	Milliamps
Mg^{2+}:	Magnesium ion
MoD:	Manner of death
NaF:	Sodium fluoride
NGS:	Next Generation Sequencing
NM:	Nanometres
PCR:	Polymerase chain reaction
PM:	Poor metaboliser
PMI:	Post-mortem interval
P/M:	Parent-to-metabolite ratio
qPCR:	Real-time polymerase chain reaction
rpm:	Revolutions per minute
RT:	Red top sample
SNPs:	Single nucleotide polymorphisms
SRM:	Salt River Mortuary
TBE:	Tris-Borate EDTA
TE:	Tris-EDTA
Tox:	Samples that underwent toxicological analysis
UCT:	University of Cape Town
UM:	Ultra-rapid metaboliser
UV:	Ultraviolet
V:	Volts
v/v:	Volume of solute over volume of solution
WC:	Western Cape
w/v:	Weight over volume (mass of solute in grams per volume of solution)
WBC:	White blood cells

Note: All gene names have been italicised whereas protein names have been left in normal font. This is to allow easy differentiation of the gene or protein version in text. This is in accordance with the guidelines for genetics in the South African Medical Journal.

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ABSTRACT

Drug usage, both of a recreational or pharmaceutical nature, is common, however the abuse of such substances is an international problem. In the Western cape, South Africa, the burden of drug-related fatalities is high compared to the rest of the country. The provincial Forensic Pathology Service may encounter cases where drug-related fatalities are unclear whether death was accidental or suicidal, or drug toxicity is inconsistent with the medical/social history. This may be due to genetic alterations with drug metabolism and it has been suggested that genetic analyses may be the next step in these cases. However, toxicology results from the National Forensic Chemistry Laboratory in the Western Cape may be delayed by months to years, meaning that upon interpretation of toxicology results, there is no chance to obtain another blood sample from the deceased individual for genetic analysis. It was therefore important to determine the suitability of blood samples collected and handled in toxicology environments for subsequent genetic tests. Previously, blood samples from 30 post-mortem cases were collected into two red-top (no additives), two grey-top (sodium fluoride/potassium oxalate) and one purple-top (EDTA) tubes. Samples from one red-top and one grey-top tube underwent toxicological analysis, followed by DNA analysis, while the remaining tubes (controls) underwent DNA analysis immediately. All samples were then stored for approximately one year, prior to this study. The DNA analysis was repeated on all blood samples ($n = 150$) and results were assessed in terms of storage time and tube type. DNA was not significantly degraded in any of the samples; however, DNA from red-top tubes had significantly lower concentrations compared to that from grey-top tubes ($p < 0.001$), regardless of whether the sample had undergone toxicological analysis. The very low yields of DNA from red-top tubes posed substantial challenges for PCR-based analysis, resulting in poor quality Sanger sequencing results. Some DNA from grey-top tubes, passed the quality assessments and hence further work is required to provide an informed decision on which tube type is better suited for genetic analyses.

CHAPTER 1: INTRODUCTION

1.1. Background

The recreational abuse of drugs is a global epidemic (Manchikanti, 2006). South Africa experienced a surge in substance-related problems in the nineties, and in 2015 the country was reported to be the major trader of illicit drugs in sub-Saharan Africa (Liebenberg *et al*., 2016) In 2009, it was reported that methamphetamine was highly abused within the Western Cape (WC) (van Heerden *et al*., 2009), but more recently cannabis and alcohol use has surged (Dada *et al*., 2014). The WC was ranked second in comparison to other provinces in the country, in terms of number of people abusing drugs (SACENDU, 2017). Local mortuaries within the province receive numerous cases of unnatural death in which drugs may have caused or contributed to death. This is especially the case at the two largest mortuaries, Tygerberg Mortuary and Salt River Mortuary (SRM) in the metropolitan region of Cape Town (CT).

SRM is one of the 17 mortuaries in WC, all run by the provincial Forensic Pathology Services (FPS) who conduct medico-legal investigations on unnatural deaths based on the National Heath Act No. 61 of 2003 (National Heath Act No. 61, 2003) and the Inquests Act No. 58 of 1959 (Inquests Act No. 59, 1959). SRM receives over 3700 admissions from the West-Metropole of CT annually. Biological specimens are collected by the forensic pathologists in cases where there is suspicion of drugs, chemicals or other substances contributing to death. These specimens usually include blood, vitreous humour, urine, bile, gastric contents and/or others. These samples currently undergo toxicological analysis by the National Department of Health's (DoH), Forensic Chemistry Laboratory (FCL) in CT, for the detection of substances that may have contributed towards the death.

The interpretation of post-mortem toxicological results is challenging due to the instability and post-mortem redistribution of drugs, and can be further compounded by an individual's genetic makeup, which may alter the way drugs are metabolised (Orphanides & Kimber, 2003). This is a major challenge in both the pharmaceutical and forensic toxicology fields, in that there is limited understanding of inter-individual differences pertaining to drug metabolism (Ingelman-Sundberg, Oscarson & McLellan, 1999). Such diversity may lead to effects including failure of therapeutic efficacy, adverse drug reactions (ADRs) and even fatality following acute and/or chronic drug use (Ingelman-Sundberg, Oscarson & McLellan, 1999).

Genetic variants in genes encoding for drug-metabolising enzymes (DME) contribute towards this variation in metabolism of drugs (Lynch & Price, 2007). This area of research has become known as pharmacogenetics. The term 'toxicogenetics' was proposed to relate to the genetic differences in metabolic pathways, which result in toxic and/or adverse effects in an individual's responses to xenobiotics. Of particular focus is the cytochrome P450 (CYPs) family of enzymes, which metabolise many drugs (Zanger & Schwab, 2013). One of these enzymes is CYP2D6, which is responsible for the metabolism of many prescription, licit as well as illicit drugs (Druid *et al.*, 1999), and forms a core component of this minor dissertation. This purpose of this chapter is to provide an overview of the literature pertaining to the background of toxicogenetics, as well as its role in forensics.

1.1.1. Toxicogenetics

Toxicogenetics is the study of heritable alterations in the genome that influence an individual's susceptibility to the adverse effects that may result from exposure to exogenous material, such as drugs (Park & Pirmohamed, 2001). Response to drug use has been reported to vary with an individual's genotype, with some people of similar age, sex and weight showing different responses to drug exposure at similar doses (Meyer & Maurer, 2014). One of the goals of toxicogenetic research is to identify genetic markers, such as single nucleotide polymorphisms (SNPs), that will predict sensitivity to drug use, as well as the role those drugs and their metabolism play in toxicity and/or death (Orphanides & Kimber, 2003). It is recognised that ADRs may occur following therapeutic or recreational drug use. The integrated role that ADRs and genetic profiles play in death however, is poorly understood.

This area of research, and particularly its application to medico-legal death investigation, is relatively new and thus is not yet part of most routine post-mortem procedures internationally, including in South Africa. Further research into this field could assist in better understanding the role of drug pharmacokinetic variability in death. A toxicogenetic result may hold value for family members as it may provide deeper understanding pertaining to the circumstances of death and understanding underlying genetic risk factors might help families find closure and acceptance of death. These results might also be directly beneficial to families, who may carry the same genetic variants as the deceased, and therefore might also be at risk for ADRs.

1.2. Cytochrome P450 enzymes: An overview

CYP enzymes are intracellular haemoproteins that activate oxygen for the molecular metabolism of lipophilic organic chemicals (Hasler *et al.*, 1999). CYPs are named due to the fact that they are bound to membranes within a cell (cyto), and contain a heme (chrome) pigment (P) that absorbs light at a wavelength of 450 nm when exposed to carbon monoxide (Nebert & Russell, 2002). CYPs are essential for the oxidation of endogenous substances and xenobiotics into more hydrophilic compounds (Nebert *et al.*, 1987). CYPs may alter the pharmacological profile of many drugs and facilitate their elimination.

Individual CYPs are grouped according to their amino acid similarities and are designated a family number, subfamily letter and a number for an individual enzyme within the subfamily (Nebert *et al.*, 1987) (Figure 1.1). There are 57 functional genes related to their synthesis in humans (Mitani & Otsuka, 2014). There are 18 families and 43 subfamilies of CYPs present in humans, with CYP2 being the biggest family (Gopisankar, 2017). Most enzymes in the CYP family are polymorphic and allelic variants resulting in altered protein activity may affect drug disposition (Zhou, 2009).

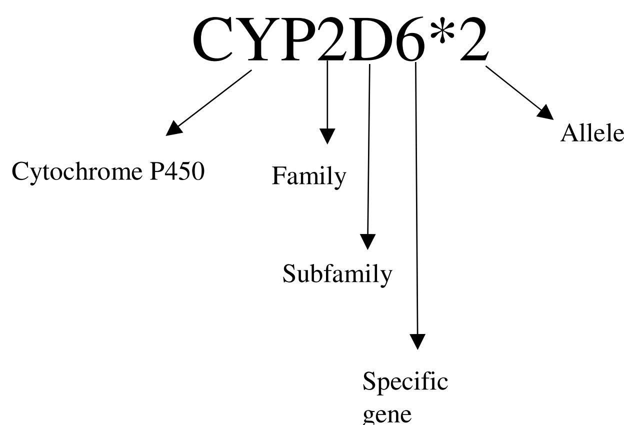


Figure 1.1: Nomenclature of CYP enzymes using CY2D6 as an example. *An example of how CYPs are named; the first three letters ('CYP') stand for cytochrome P450, followed by a number (e.g. '2') which represents the family the enzyme belongs to. The letter after the family depicts a subfamily (e.g. 'D'), then the specific gene (e.g. '6'). Any number after an asterisk represents an allele.*

1.2.1. Factors affecting cytochrome P450 expression and function

Various factors influence the function and expression of CYPs, such as an individual's demographics and their genetic makeup. An individual's sex has been reported to influence the expression of DMEs (Gandhi *et al.*, 2004). Higher levels of protein expression were found to be higher in females than in males, suggesting that women may metabolise some drugs faster than men (Gandhi *et al.*, 2004). This was widely studied for CYP3A4 but evaluation of sex in other members of the CYP family, including CYP2D6, remains to be fully investigated.

Age was also an established factor affecting drug metabolism. Drug metabolism is reduced at the extremes of life (Zanger & Schwab, 2013). In infants, this is due to immaturity of CYP enzymes, which only become fully developed during the first year of life. In the elderly, drug metabolism is reduced, however this may be due to low expression or activity of enzymes (Zanger & Schwab, 2013). It has been suggested that reduced rates of metabolism may be due to inhibition of enzymes because of drug-drug interactions (Cotreau, von Moltke & Greenblatt, 2005). Organ dysfunction of liver and kidneys, which may also increase with age, could also affect drug metabolism and elimination (Musshoff *et al.*, 2010).

Genetic variants have also been associated with CYP function. The term 'genetic variant' refers to the occurrence of two or more alleles at a particular locus (Ismail & Essawi, 2012). Genetic variants in DMEs are common occurrences and are one of the main causes of inter-individual variation of drug effects (Meyer & Zanger, 1997). This inter-individual variation results in distinct subgroups in the population that vary in their ability to undergo biotransformation. Variants in the genes of the enzymes result in increased, decreased or absent enzyme expression or activity (Meyer & Zanger, 1997). CYP2D6 affects the metabolism of over 35 clinically used drugs and these include opioids, antidepressants, neuroleptics and antiarrhythmics (Meyer, 1996). CYP2D6 is a key enzyme in the metabolism of many commonly used medicinal and recreational drugs, many of which are detected in forensic toxicological screening.

1.2.2. CYP2D6

The hepatic enzyme CYP2D6, is vital in the metabolism of certain neuroleptic, antiarrhythmic, tricyclic antidepressant and B-adrenoceptor blocking agents (Lennard, 1990). The *CYP2D6* gene is highly polymorphic, giving rise to an enzyme with variable function. Over one hundred

allelic variants of CYP2D6 have been identified, which had either fully functional, reduced function or non-functional alleles (Kiss *et al*., 2018). These alleles resulted in four phenotypes defined for all CYPs: poor metabolisers (PM), ultra-rapid metabolisers (UM), intermediate metabolisers (IM) and extensive metabolisers (EM) (Figure 1.2). An individual's most functional *CYP2D6* allele predicts the phenotypic activity. For example, an individual with PM and EM alleles will have EM activity (Owen *et al*., 2009).

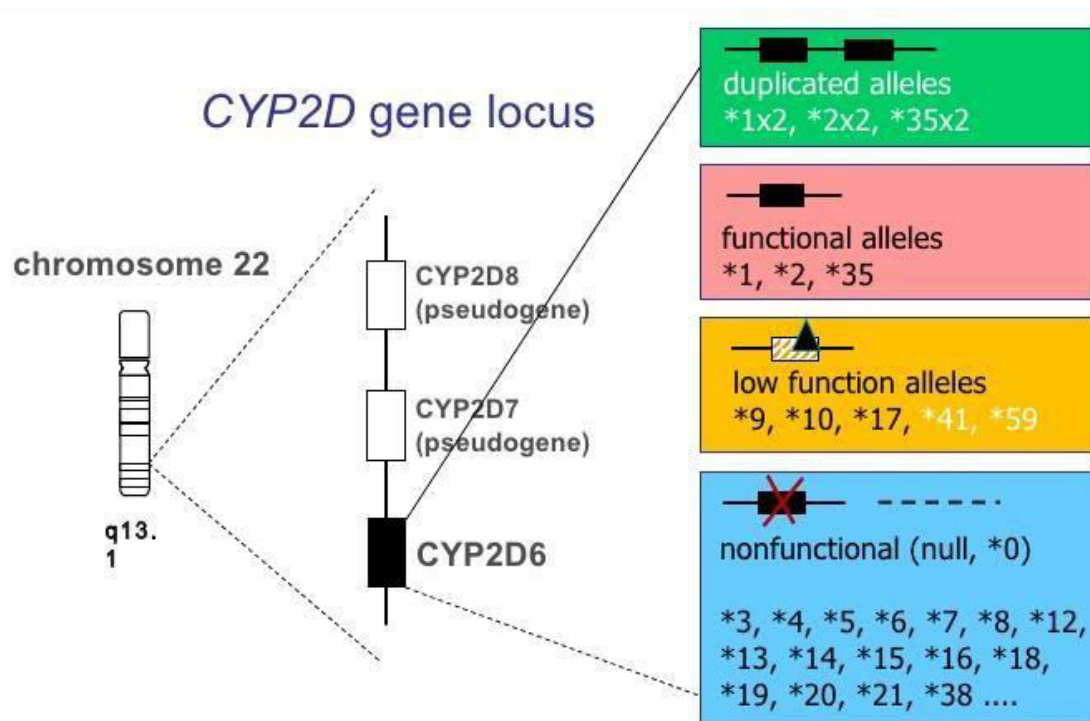


Figure 1.2: Illustration of the *CYP2D6* gene and the allelic variants. *The duplicated alleles (green box) represent the UMs, the functional alleles (pink box) represent the EMs. The yellow and blue boxes represent IMs and PMs respectively (Goetz, 2009).*

The CYP2D6 enzyme is a polypeptide of 497 amino acids, with a high affinity for alkaloids (Zanger, Raimundo & Eichelbaum, 2004). It was reported that the expression of the enzyme is different to other hepatic cytochrome P450 enzymes, as CYP2D6 was not regulated by any environmental factors or inducible by any known hormones (Ingelman-Sundberg, 2005). According to Ingelman-Sundberg (2005), between 20 to 25% of clinically important drugs are metabolised in part by CYP2D6 and individuals without functional *CYP2D6* genes may metabolise certain CYP2D6 substrates at a lower rate, increasing risk for ADR. *CYP2D6* genotyping in post-mortem forensics may provide essential information on whether a variant

may have contributed to a drug-related fatality (Sistonen *et al*., 2005).

The *CYP2D6* gene is located on chromosome 22q13·1 (Eichelbaum *et al*., 1987), spans a 4.2 kb region, and belongs to a cluster of the pseudogenes *CYP2D7* and *CYP2D8* (Sistonen *et al.*, 2005). The gene encompasses nine exons with an open reading frame of 1383 bp coding for 461 amino acids (Cascorbi, 2003). The most important genetic variations in terms of predicting phenotypic activity are splice-site variations, premature stop codons and/or large deletions, which resulted in complete loss of enzyme function (Cascorbi, 2003).

Reports have illustrated that drug toxicity either causing or contributing to death, were likely due to ineffective CYP2D6 metabolism and elimination of drugs, together with drug-drug interactions (Koski *et al*., 2007). On the contrary, different studies were undertaken to determine the impact of studying genetic variants in routine forensic cases. Genotyping of *CYP2D6* for citalopram metabolism indicated that drug-drug interactions were more important than metabolic deficiency (Holmgren *et al*., 2004). Despite the contradicting results, genotyping of *CYP2D6* genes remains important, as genotyping of DNA could be used in the interpretation of complex and/or contradictory drug-toxicity cases (Levo *et al*., 2003). The following section describes what forensic toxicology entails and the complexities involved with interpretation of results, particularly as they relate to post-mortem cases.

1.3. Forensic toxicology

Forensic toxicology is a discipline aimed at assisting in the detection, identification and quantitation of drugs, chemicals and other xenobiotics in human biological materials, and the interpretation of those results for medico-legal purposes (Maurer, 2007). Forensic toxicology is applied in numerous areas including post-mortem toxicology, human performance toxicology (driving under the influence of alcohol and/or drugs), child welfare and drug-facilitated crimes (Ojanperä, Kolmonen & Pelander, 2012). Analyses and interpretation of drugs in post-mortem cases can be particularly challenging, due to the interdisciplinary knowledge and complex skillset required, particularly within analytical chemistry, toxicology and pathology. One of the vital issues with interpretation of post-mortem toxicological results is the likelihood of changes in drug concentration after death (Ferrara *et al*., 2017). The variations in drug concentration are a result of degradation of analytes, putrefaction,

redistribution and analytical interference with matrices (Skopp, 2010).
Blood is most routinely collected at post-mortem because it provides insight into the drugs that were in the system and therefore may be able to tell something about the role of drugs in impairment. However, many pre-analytical factors such as drug stability, re-distribution, sample handling and storage require consideration when analysing and interpreting results. Given these, blood samples for toxicological analysis are usually collected into sodium fluoride (NaF) preservatives (2% w/v) to prevent enzymatic activity and are then refrigerated or frozen. Blood samples may also be collected without preservatives if it is suspected that the preservative may interfere with the analytes and results. Additional samples such as vitreous humour, urine, and gastric contents may also be collected, depending on the interpretive requirements in the case.

Collection of samples should be as close to time of death as possible, as drugs tend to degrade with time (Dinis-Oliveira *et al.*, 2010). Isolation of chemicals or drugs from post-mortem matrices is difficult because of the wide range of specimens encountered (Ferrara *et al.*, 2017). Other factors to be considered in post-mortem specimen collection and analysis include how and when the drug was administered, history of previous drug use, drug-drug interactions and the likelihood of finding the drug in particular specimens (Dinis-Oliveira *et al.*, 2010). Aspects such as sampling site(s) and time-related differences in post-mortem drug concentrations also have to be taken into consideration when interpreting results (Musshoff *et al.*, 2010). In addition, individual pharmacokinetics and/or pharmacodynamics, together with tolerance to drugs increases the complexity of interpreting analytical results (Ferrara *et al.*, 2017). This in turn makes post-mortem toxicology one of the most challenging disciplines within the forensic toxicology field.

Post-mortem toxicology may assist forensic pathologists in determination of cause and manner of death (CoD and MoD, respectively) (Druid *et al.*, 1999). Previously, a high parent to metabolite (P/M) ratio of a drug was used to distinguish an acute overdose (Druid *et al.*, 1999). However, P/M ratios may provide false evidence as low ratios may be encountered in acute overdoses (such as in delayed deaths), or high ratios may be encountered as a result of reduced metabolism of the parent drug (Nemeroff, Devane & Pollock, 1996). Besides P/M ratios, toxicity can also be inferred from post-mortem toxicological results based on contextualisation of the case situation. While concentrations of drugs may be suggestive of toxicity; challenges may arise in determining the circumstances (and even sometimes the cause) of death if the

results are inconsistent with history of use or other case information. This may be important in the court's ultimate determination of the MoD (accident, natural, suicide, homicide or undetermined).

Genotyping has been reported to assist in such cases and examples include cases involving tramadol and its metabolites, found in 33 post-mortem cases (Levo *et al.*, 2003), as well as oxycodone and its metabolites detected in 15 post-mortem cases (Jannetto *et al.*, 2002). In both studies it was hypothesised that drug fatalities were partly due to variants in *CYP2D6.* The results showed a correlation between genotype and drug concentrations, suggesting that genotyping was useful in some cases by providing a more definitive interpretation of drug toxicity. Other variables such as drug-drug interactions might need to be investigated as that might assist in determining why ADRs took place (Musshoff *et al.*, 2010).

1.4. Toxicogenetics in casework

Toxicogenetic testing has also been applied to other cases and has resolved or added value in several instances. For example, a study by Sallee, Devane & Ferrell (2000) was the first to demonstrate how *CYP2D6* genotyping was useful in explanation of post-mortem toxicology results. In the case, a 9-year-old boy diagnosed with extreme behavioural problems and was treated with a mixture of psychotherapeutic drugs over a period of 10 months, which included three hospitalisations (Sallee, Devane & Ferell, 2000). The boy reportedly died of fluoxetine intoxication and very high concentrations of the drug was found during post-mortem toxicological analysis. A genetic analysis revealed that the deceased had a totally defective *CYP2D6* gene*,* which resulted in poor metabolism and elimination of fluoxetine. It was suggested that fluoxetine concentrations had accumulated with therapeutic administration, thus resulting in drug toxicity and death.

Another case involved a baby who was found dead 13 days after birth (Koren *et al.*, 2006). The baby's post-mortem did not show any anatomical anomalies, but a blood concentration of morphine of 70 ng/ml was reported (Koren *et al.*, 2006). Investigations showed that the baby was breastfed by a mother who was receiving codeine. Genotyping was performed on *CYP2D6,* as the CYP2D6 enzyme is responsible for the codeine metabolism to active morphine. The mother was found to be heterozygous for *CYP2D6*2A,* which is classified as a UM. The infant had two functional alleles classified as extensive metaboliser. The mother's genotype led to

increased formation of morphine from codeine. The case showed how *CYP2D6* variants even in mothers, could be life-threatening for babies who are breastfed. This case illustrated how genetic analysis may assist to determine why there was morphine in the child and ultimately the circumstances and MoD.

In a separate report, it was shown how a *CYP2D6* variant was linked to the death of a 43-year-old man due to drug toxicity (Koski *et al.*, 2007). The man was found dead on the floor with medicinal drugs including fluoxetine, disulfiram and diazepam next to him. Toxicology samples taken at autopsy revealed 2.4 mg/L of doxepin and 2.9 mg/L nordoxepin in blood, with a ratio of 0.83 between the two and the cause of death was determined to be fatal doxepin toxicity. To interpret the doxepin to nordoxepin ratio, *CYP2D6* and *CYP2C19* genotyping was carried out as doxepin is metabolised by CYP2C19. The results revealed a complete absence of CYP2D6 enzyme and extensive metabolism in terms of *CYP2C19*. In a parallel study there were 20 cases of fatal doxepin poisoning and the doxepin to nordoxepin ratio was at least 3.8 (Koski *et al.*, 2006). In the doxepin poisoning case reported above, a defective *CYP2D6* genotype was reported to coincide with the high concentration of nordoxepin found and was denoted as contributory to death.

The case examples highlighted above illustrate that toxicogenetic analyses in a post-mortem forensic setting may assist in revealing new information on CoD and/or MoD. However, toxicogenetic analyses requires considerations associated with both toxicological and genetic analyses and are thus not always straightforward.

1.5. Post-mortem procedure and collection tubes

The current local post-mortem procedures of unnatural deaths in the WC do not routinely include genetic testing. This means that in cases where the CoD and/or MoD is ambiguous following ancillary analyses, and where genetic testing maybe the next step, no suitable sample was obtained at autopsy for DNA testing. Toxicology testing however, is fairly routine in the local post-mortem procedure, whereby blood samples are collected into vials containing specific chemicals to preserve the integrity of drugs. These include containers such as grey-top tubes, which contain 1-2% w/v NaF as a preservative to prevent bacterial or enzymatic reactions, and potassium oxalate ($C_2K_2O_4$) as an anticoagulant (De Martinis *et al.*, 2004).

Plain vacutainers (red-top tubes), containing no preservatives or additives (Penetar *et al*., 2008), are also used to collect blood samples where the additives of the grey-top tubes would affect the toxicological results. Both of these tubes (red and grey-top) are designed for the collection of samples for toxicological and other biochemistry analyses, and not genetic analyses, as DNA degrades without suitable preservation (Lahiri & Schnabel, 1993).

For genetic analyses, blood is typically collected into purple-top tubes which contain ethylenediamine tetra-acetic acid (EDTA). EDTA binds calcium ions and thus blocks the coagulation of blood, providing an optimum environment for DNA preservation in the short term, or for longer periods if stored correctly. It was reported that DNA of good quality could be extracted from whole blood stored at 4 ℃ for one month in EDTA-containing tubes (Nederhand *et al*., 2003). Another study reported that EDTA inhibited the degradation of DNA when compared to other anticoagulants, showing that EDTA in the collection tube provided an optimal environment for DNA (Lahiri & Schnabel, 1993). Storage time also played a role in stability of DNA, whereby whole blood samples in purple-top tubes stored at 4 ℃ for one year showed a decrease in DNA yield over time (Bulla *et al*., 2016).

However, due to the post-mortem procedures in the local context, it is not feasible to obtain blood in a purple-top tube for every case and store it for months to years, in case a toxicogenetic analysis is subsequently needed – which would ultimately be in the minority of cases. Currently, the system of toxicological analyses in the country is limited to three National Health Forensic Chemistry laboratories. A systematic backlog has developed over time, rendering the turnaround time for toxicological results variable – but usually months to years. If case results come back after a longer time and genetic testing may be required, it is important to ascertain whether the blood obtained for toxicological analyses (*i.e.* stored in red and grey-top tubes) and analysed in a DNA-uncontrolled laboratory, would subsequently be suitable for genetic testing.

The NaF preservative contained in the grey-top tubes has been reported to induce DNA damage even though the mechanism is unknown (Podder *et al*., 2015). Fluoride intoxication produced specific metabolic alterations in nucleic acid synthesis in the livers of experimental rabbits (Jha, Shah & Verma, 2012). The conclusion was that there was reduced DNA in fluoride-containing cultured cells. A study was carried out on the effects of fluoride on *Vigna radiata* seeds and showed that there was an increase in DNA damage as fluoride concentrations

increased (Agarwal & Khan, 2016). Not only is the presence of NaF a factor that affects DNA, but the preservative expires after a certain amount of time. It is unclear what affect this would have on DNA recovery once the NaF tubes have expired; however, this investigation was outside the scope of this study. The red-top tubes with no preservatives were shown to yield DNA of high quality after storage at 4 ℃ for sixty days (Lahiri and Schnabel, 1993). To the author's knowledge, there is no published data illustrating the direct effects of $C_2K_2O_4$ on DNA.

To investigate if post-mortem blood samples taken for toxicology could subsequently be used for genetic analysis (if necessary), a pilot study was recently undertaken in our research group (Vuko, 2017). The quantity, quality and purity of DNA from thirty cases of suspected drug-related fatalities was assessed, and it was observed that post-mortem blood samples collected into red-top tubes (no preservatives) were suitable for DNA analysis (including DNA extraction, polymerase chain reaction (PCR) and Sanger sequencing). This contrasted with blood collected into grey-top tubes, which showed low signal and chemical artefacts during Sanger sequencing (in-house data). The previous pilot study investigated the integrity of DNA 16 weeks after collection at autopsy, however in South Africa, there is usually a greater time delay between autopsy and government toxicological analyses. Therefore, there is a need to explore the effects of storage in these tubes over a longer period of time to better understand DNA quality in a locally-relevant situation.

1.6. Rationale

In South Africa, determining CoD during post-mortem is one of the main functions of the provincial FPS. This is not always straightforward in cases where the individual died in a sudden and unexpected manner and drug involvement is ambiguous. In such cases, or in at least a subset thereof, death may be linked to underlying variations in the way humans metabolise drugs. One way to test for this phenomenon would be to examine the sequence of the specific genes encoding the enzymes that are involved in the metabolism of the implicated drug(s) (Musshoff, Stamer & Madea, 2010). This may assist in identifying if the deceased had a genetically altered metabolism, which induced drug toxicity or reduced the therapeutic efficacy of a drug. The present post-mortem procedure does not make provision for such toxicogenetic testing.

For such a test to be included in the FPS procedure, it must first be ascertained whether the blood already/routinely collected at autopsy, will have usable DNA to perform genetic analysis after the sample has undergone toxicological analyses and after long-term storage. If not, then this motivates for the need to collect an extra blood sample in these situations, specifically for DNA analysis, so that suitable biological analyses can be carried out if it is deemed necessary downstream.

One may suggest that blood should routinely be collected into purple-top tubes or onto FTA cards during post-mortem; however, this would put more pressure on the already resource-constrained environment with exceptionally high caseloads. There is also a greater delay between autopsy and toxicology analysis in South Africa compared to other countries. It was reported that CT and Johannesburg had a backlog of 2878 and 7843 cases, respectively, by February 2016, Pretoria had 5013 by January of the same year (Evans, 2016). The national backlog was alleged to translate to 5 to 8 years waiting period for toxicology results (Raphaely, 2011). This study therefore aimed to determine whether blood stored in NaF/$C_2K_2O_4$ or without preservatives (grey and red-top tubes respectively) may be used for subsequent genetic analyses (when warranted).

Information collected from this study will assist the pathologists in forensic post-mortem investigations, to make an informed decision on whether blood samples collected for toxicological testing can later be used for genetic analysis, or if such samples should be collected separately, in the case that future testing is warranted.

1.7. Aim

To assess the quantity and quality of DNA from blood samples, which were collected in tubes with NaF/$C_2K_2O_4$ (grey-top tubes) and tubes without preservative (red-top tubes) for toxicological screening and stored for one year.

1.8. Objectives

To this end, the objectives were to:

- Extract DNA from blood samples of 30 cases collected in 2017 which were then stored for one year.
- Assess and compare the quality and quantity of DNA obtained from red, grey and purple-top tubes using a variety of molecular methods.
- Apply statistical analysis tests to assess the suitability of samples for DNA analyses compared to results generated one year ago.

CHAPTER 2: METHODS AND MATERIALS

2.1. Study population

The study comprised a cohort of 30 cases admitted to SRM between 1st May 2017 and 30th August 2017. Informed consent was obtained from each deceased individual's next-of-kin, aligned with an established ethical framework (Heathfield *et al.*, 2017) and is also described in detail elsewhere (Vuko, 2017). This study received ethics approval from the University of Cape Town (UCT) Human Research Ethics Committee (HREC/REF: 110/2017).

2.1.1. Inclusion and exclusion criteria

Cases selected for this study were those where drugs may have caused or contributed to death, or where there was a history of drug use prior to death. The cohort consisted of individuals from African (n = 14), European (n = 4), Asian (n =1) and mixed (n = 11) ancestries. There were 22 males (age range 22-66) and 8 females (age range 21-57). Cases where the body was found in a decomposed state were excluded, as well as cases where the decedent was below the age of 18 years. Full details of the inclusion and exclusion criteria were described previously (Vuko, 2017).

2.2. Sample collection

For each of the 30 cases, blood samples were collected at autopsy into five tubes: 4 ml blood into one purple-top tube (BD Tube, New Jersey, USA), 4 ml blood into each of two grey-top tubes (SG Vac, Johannesburg, SA), and 4 ml blood into each of two red-top tubes (SG Vac, Johannesburg, SA).

The grey-top tubes contained $NaF/C_2K_2O_4$, while the red-top tubes had no additives or preservatives. Blood specimens collected for toxicological analysis at SRM are commonly collected in these containers and thus were the focus of this study. The purple-top tubes containing EDTA were used as controls as they are standardly used for genetic analyses. A unique identifier code was assigned to each case during the pilot project to maintain confidentiality and to track blood samples throughout this study.

2.3. Brief description of the 2017 pilot project

After sample collection, one red-top and one grey-top tube from each case were designated R-Tox and G-Tox respectively. These samples were taken through toxicology laboratory analysis as described previously (Vuko, 2017). The remaining red-top and grey-top tubes did not undergo toxicological analysis and were thus designated R-No-Tox and G-No-Tox. The purple-top tubes also did not undergo toxicological analysis and were designated P-No-Tox. All samples underwent DNA extraction as well as DNA quality and quantity assessments (Figure 2.1). Full details of these procedures are described in the Master's dissertation by Vuko (Vuko, 2017). After this analysis, samples were stored at 4 ^{o}C until this follow up study.

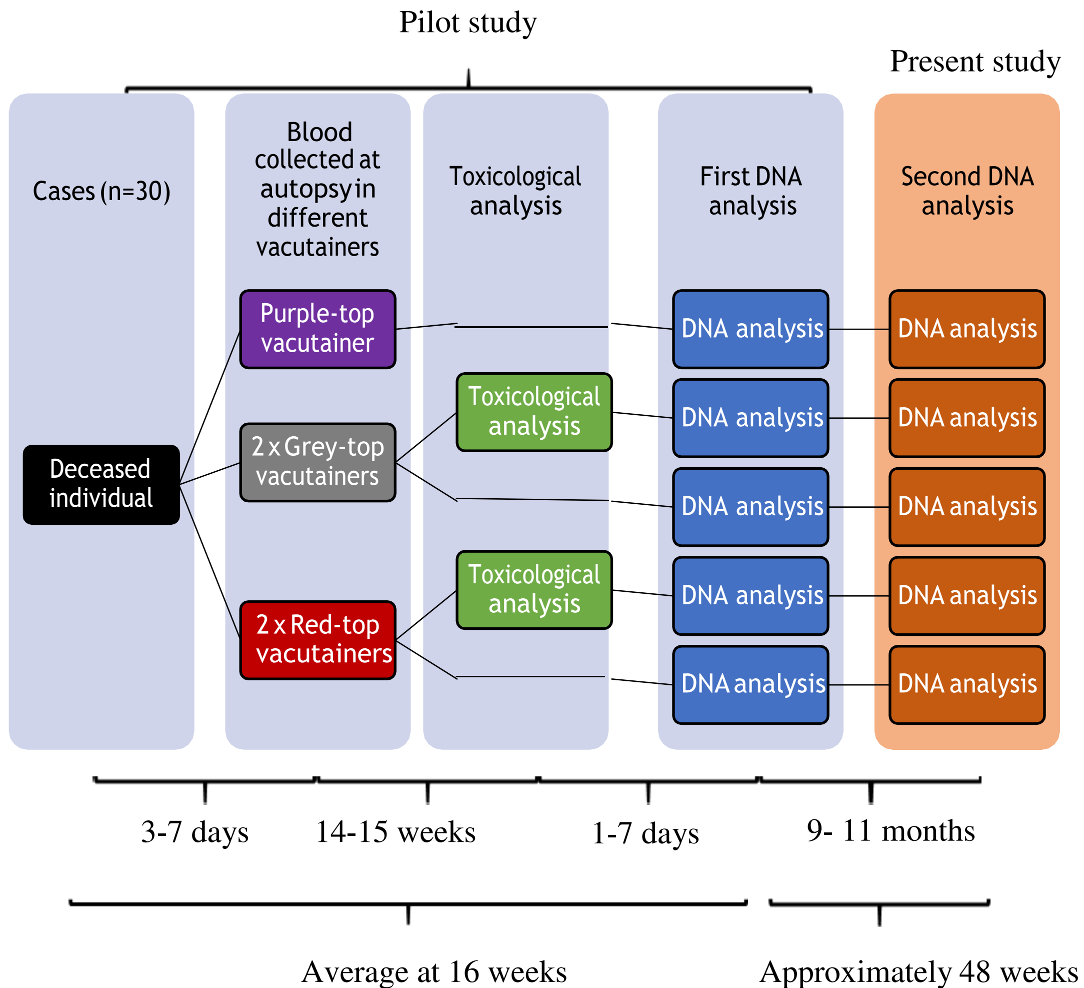


Figure 2.1: Illustration of sample collection and subsequent analyses during the pilot project and the current study. *The figure shows laboratory procedures after post-mortem sample collection, during the pilot and present study. During the post-mortem at SRM, 4ml x 5 blood samples were collected into 2 red-top tubes, 2 grey-top tubes and 1 purple-top tube respectively. R-Tox and G-Tox samples were kept in the UCT Forensic Toxicology Laboratory until DNA extraction. The rest of the blood samples underwent DNA extraction after post-mortem and were kept in the UCT Molecular Forensics' laboratory. Sample storage was at 4 ºC throughout the studies.*

2.4. Laboratory procedures for the present study

2.4.1. DNA extraction

After approximately 48 weeks following the pilot study, the same blood samples were retrieved from storage and DNA was extracted from all samples. This was performed using the QIAamp®

DNA Investigator kit (Qiagen, Hilden, Germany), with slight modifications to the manufacturer's protocol. The same modifications were applied during the pilot study (Vuko, 2017) as follows: for the procedure, 100 µl of blood was eluted into 50 µl of ATE buffer and centrifuged at 10 000 rpm for 1 minute and 30 seconds instead of 1 minute. To maximise DNA recovery, the elution step was repeated with another 50 µl of ATE buffer, which meant that there were two samples of DNA obtained for each tube. The controls used were molecular biology grade water (Thermo Fisher Scientific, Massachusetts, USA) stored in purple, grey and red-top tubes, which underwent the same DNA extraction procedure as the blood samples.

2.4.2. DNA quantification

2.4.2.1. Quantity and purity assessment of DNA by Nanodrop spectrophotometry

Total genomic quantification of the DNA in this study was performed using the Nanodrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA). According to the Beer Lambert law, there is a direct correlation between absorbance and concentration where absorbance (A) = extinction coefficient (Ɛ) x concentration (c) x path length (l) (Desjardins *et al.*, 2009). To measure DNA concentration, the instrument was first blanked by placing 2 µl of elution buffer ATE (Qiagen, Hilden, Germany) onto the instrument's pedestal, followed by 2 µl of each sample thereafter. The results were recorded from the Nanodrop™ 2000/2000c software (Thermo Fisher Scientific, Massachusetts, USA). The quantity of DNA as measured by Nanodrop™ spectrophotometry was obtained to determine if samples needed dilution prior to PCR-based assays downstream.

To assess purity of the DNA, the absorbance ratios 260/230 and 260/280 were measured by the Nanodrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA). $A_{260/280}$ is used to determine protein contamination of nucleic acids, while $A_{260/230}$ shows the presence of organic compounds such as aromatic compounds, chaotropic salts and phenol (Desjardins, Hansen & Allen, 2009).

2.4.2.2. DNA quality assessment by real-time PCR

Isolated DNA was quantified by real-time PCR (qPCR) to assess the quantity of amplifiable DNA, degradation of DNA, as well as cycle threshold (Ct) for the internal positive control

(IPC). This was performed using the Quantifiler® Trio DNA Quantification kit (Thermo Fisher Scientific, Massachusetts, USA) according to the manufacturer's instructions. DNA was quantified and amplified using the 7500 Real-Time PCR system (Applied Biosystems, California, USA).

The Quantifiler® Trio DNA Quantification kit (Thermo Fisher Scientific, Massachusetts, USA) simultaneously quantified total human and male DNA in a single reaction. The targets of interest were one large autosomal region (214 bp), small autosomal region (80 bp) and one Y chromosome region. The assay measures degradation, given as a degradation index (DI), which was calculated by dividing the concentration of small autosomal target by the concentration of large autosomal target (Köchl *et al*., 2005). A DI less than or equal to one indicates that DNA is not degraded, as amplification was similar between different sized targets. In contrast, larger DI indicates that the smaller target could amplify more than the large target, which would indicate fragmentation (*i.e.* degradation) of the DNA. The result from the quantification of the Y target was used as an internal quality measure to confirm if DNA amplified was from a male.

The IPC in the Quantifiler® Trio DNA Quantification kit (Thermo Fisher Scientific, Massachusetts, USA) is a synthetic DNA template and primer set, which provides positive confirmation that all assay components are working correctly. The IPC is also useful in identifying samples containing inhibitors. In real-time PCR, a positive reaction is detected by accumulation of a fluorescent signal and Ct is defined as the number of cycles for the fluorescent signal to cross the threshold (*i.e.* exceed background level) (Ng *et al.*, 2009). Ct values are inversely proportional to the quantity of nucleic acid in a sample, which means lower Ct values would indicate less inhibitors in a sample. Therefore, Ct for the IPC of red and grey-top samples was assessed to determine if there were PCR inhibitors present, and if this parameter changed over time.

2.4.2.3. Application of genetic analysis

The purpose of the study was to determine whether blood samples that were collected for toxicological analysis could be used for DNA extraction and genetic analysis thereafter, after storage for one year. Therefore, the ultimate test for the suitability of samples for subsequent genetic analysis was to apply a molecular assay to the DNA, similar to that which would be used in a toxicogenetic analysis. Given that many drugs are metabolised by CYP2D6, the

CYP2D6 sequence, which has nine exons, was examined. Primers that amplify these exons had been previously designed (Vincent, 2017) and are shown in Appendix A.

One case (number 22) was prioritised for this analysis, because results from toxicological analysis showed presence of drugs metabolised by CYP2D6. As such, the genetic results would, hypothetically, be of value to such a case (provided the assay was suitably validated). The same case was selected during the pilot study, which also facilitated the comparison of results. DNA from the red-top and grey-top tubes that had undergone toxicological analysis were used for genetic analysis, both during the pilot and this study.

2.4.2.4. Amplification of *CYP2D6* exons

Following Sanger sequencing during the pilot study, variations in *CYP2D6* were discovered in exons 1, 4, 8 and 9 for case 22 (Vuko, 2017). Therefore, PCR was performed for each of these exons in the present study to verify if these variants could still be observed one year later. To this end, 10 µM of the forward and reverse primers (Integrated DNA Technologies, Iowa, USA) were added to 2X GoTaq Green Master mix (Promega, Wisconsin, USA) to amplify DNA (maximum volume of 5 µl) in a total volume of 25 µl.

The samples underwent PCR in the BioRad T100 thermal cycler with the following conditions: initial denaturation for 5 minutes at 95 ºC; 30 cycles of denaturation at 95 ºC for 30 s, annealing at 62.9 ºC (for primers A, C, and I) or 55.3 ºC (for the remaining primers) for 30 s, elongation at 72 ºC for 30 s; and final extension at 72 ºC for 5 minutes.

Agarose gel electrophoresis was used to determine if amplification of the targets was successful. Agarose powder (Lonza, Basel, Switzerland) was dissolved into 1X Tris-Borate EDTA buffer (TBE) and 0.01% (v/v) of SYBR® Safe DNA gel stain (Thermo Fisher Scientific, Massachusetts, USA) was added to the mixture to prepare a 1% (w/v) agarose gel. A volume of 2 µl of each sample was loaded into the wells, alongside a 1 Kb Plus DNA ladder (Thermo Fisher Scientific, Massachusetts, USA). Electrophoresis was performed for 60 minutes at 80 V and 400 mA.

In this follow-up study, after PCR, it was noted that amplification of DNA from the red-top tubes was poor, and this was attributed to the low yields of DNA obtained. As such, PCR was

repeated using the PCR product as the template for a secondary PCR for each region. In the secondary PCR, the extension time was increased to 1 min, and cycles increased to 40 for exon 1.1 and 8.

2.4.2.5. Sanger sequencing

Sanger sequencing was performed to determine the nucleotide sequences of the amplified products, in both the forward and reverse directions. Post-PCR products were cleaned using the Nucleofast® 96 PCR Clean-up kit (Macherey-Nagel, Düren, Germany) to remove unincorporated primers and dNTPs. Sequencing was performed using the BigDye® Terminator v3.0 cycle kit (Applied Biosystems, California, USA) at the Central Analytical Facility at the University of Stellenbosch, using their in-house protocol.

The sequences were viewed using Chromas Lite software version 2.6.5 (Technelysium, South Brisbane, Australia). The attained sequences were compared to those from the pilot study, as well as to the reference *CYP2D6* sequence which was obtained from the Ensembl genome browser Release 93 (http://www.ensembl.org; accessed 23 July 2018). Alignment was done using Sequence Alignment Editor, BioEdit version 7.2.6 using ClustalW with 1000 bootstrap.

Electropherograms (EPGs) were defined as unusable if the calling of nucleotides stopped mid-sequence and could not be aligned with the reference sequence. The quality was defined as good if all peaks were called, noise to signal ratio was low and could be aligned to the reference sequence. Fair was if sequencing in one direction was good and poor in the other direction, despite repeating of the poor, and poor quality was if the noise to signal ratio was high causing incorrect base pair calling and/or with the distinct presence of chemical artefacts.

2.5. Data analysis

The data produced was stratified into three groups, according to the type of tubes that the samples were collected in: grey, red and purple-tops. Data from the grey and red-top tubes were further divided into two categories: Tox and No-Tox samples. Data analysis was performed using Stata® Data Analysis and Statistical Software (StataCorp, Texas, USA).

The Shapiro Wilk test was first performed to assess the distribution of data. If there was normal distribution, then a one-tailed student's t-test was used to assess significant differences between the median values of the compared datasets (i.e. concentration, DI and purity ratios). If there was a non-normal distribution, then the one-tailed Wilcoxon Signed Rank test used to assess these differences. In both cases, the Bonferroni's post hoc test was applied to correct for multiple testing.

Since it was found that for all variables (i.e. concentration, DI and purity ratios), data from test Tox and No-Tox samples for a specific tube type were not significantly different, these data were combined for red and grey-top tubes, respectively, for multiple regression analysis. This test was performed in attempt to model the relationship between (i) time since blood sample collection at autopsy, (ii) tube type (red and grey), and (iii) concentration or DI or purity ratio. Heteroscedasticity was analysed by residual versus fitted plots, and the Breusch-Pagan test and robust methods were used to counteract low heteroscedasticity. This test is used to assess whether the variance of the error of terms is dependent on the independent variable, which in this case, was tube type. The level of significance (□) was 0.05.

CHAPTER 3: RESULTS

3.1. Introduction

DNA was extracted from the stored blood samples (n = 150) after 48 weeks of storage; *i.e.* from all five collection tubes from all 30 cases. After extraction, the quality and quantity of DNA was assessed using qPCR. Data were sorted according to (i) collection tube type and (ii) analyses performed *i.e.* R-No Tox, R-Tox, G-No-Tox, G-Tox and P-No-Tox. Median values of DNA concentration (ng/µl), purity ratios and DI were calculated to represent the data. Appendix B shows the summarised data.

3.2. DNA concentration

DNA quantification after 48 weeks by qPCR showed that blood samples from G-No-Tox had the highest median DNA concentration of 37 ng/µl, followed byG-Tox samples with 21 ng/µl (Figure 3.1). The difference between the grey-top tubes (G-No-Tox and G-Tox) was however not significant ($p = 0.05$). The red-top tubes had the lowest median concentration with 0.8 ng/µl (R-No-Tox and R-Tox). Comparison between R-No-Tox and R-Tox (intra-tube) showed that there was no significant difference in qPCR concentration ($p = 0.87$). The purple-top tubes had a concentration of 7.5 ng/µl and this was significantly higher than the red tubes ($p = 0.01$).

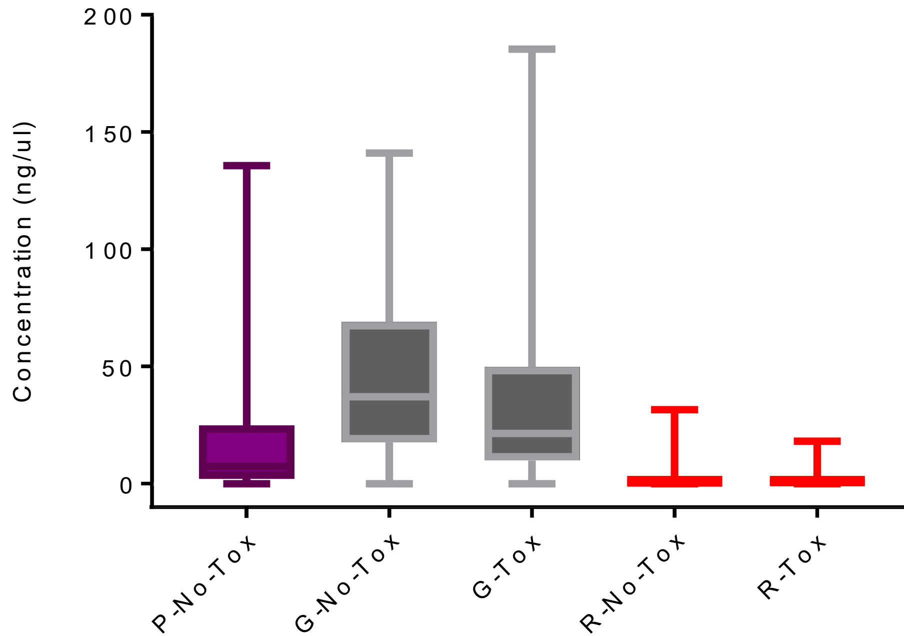


Figure 3.1: DNA quantification after 48 weeks by qPCR. *Blood samples were collected into five vacutainers: two red, two grey and one purple-top. The samples were assessed for DNA quality and quantity and stored for approximately 48 weeks at 4 °C. After 48 weeks, DNA was extracted from all blood samples using the QiaAmp® DNA Investigator kit (Qiagen, Hilden, Germany), followed by DNA quantification.*

There was no significance difference in terms of DNA concentration between tubes of the same colour *i.e.* R-No-Tox vs R-Tox and G-No-Tox vs G-Tox and therefore the results for Tox and No-Tox samples for each tube type were combined for further statistical analysis. DNA concentration was assessed over time for red-top and grey-top samples and, as expected, the DNA concentration decreased over time for red-top samples, and this correlation was significant ($r = -0.24$, $p < 0.001$) (Figure 3.2). However, DNA concentration appeared to significantly increase with time for grey-top samples ($r = 0.21$, $p = 0.017$), which was unexpected. A linear regression analysis, which modelled concentration based on tube type and time in weeks (*i.e.* time between blood collection at autopsy and DNA extraction), showed that time and tube type accounted for 14% of the variance in concentration ($p < 0.001$). The following equation was established to model DNA concentration based on these two variables:

DNA concentration = 0.74 (tube type) + 0.019 (weeks between blood collection at autopsy and DNA extraction) + 0.25

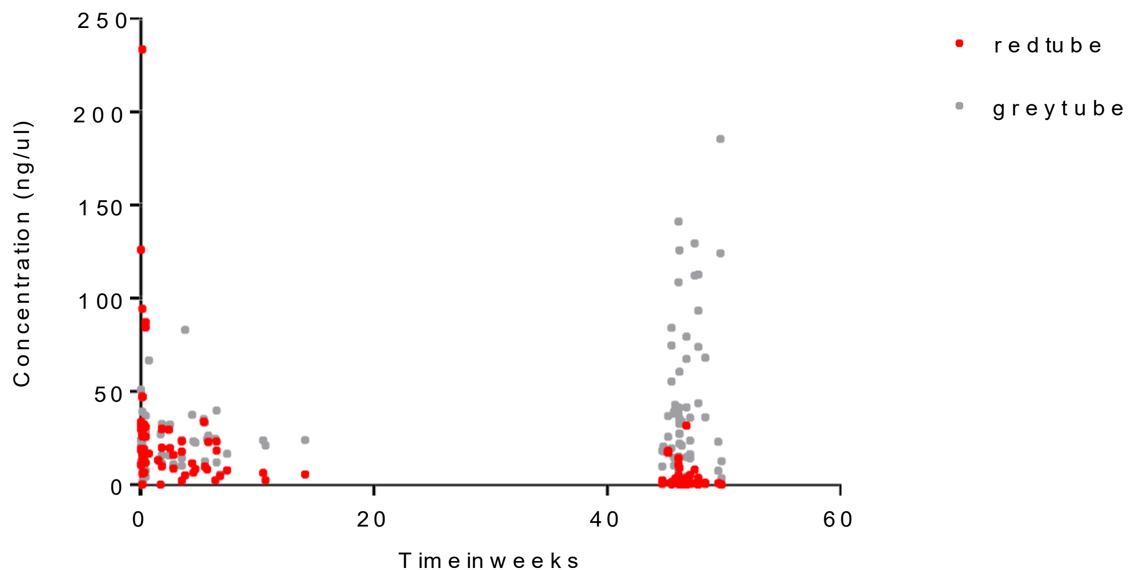


Figure 3.2: DNA concentration over time. *DNA concentration for red and grey-top tubes was combined for Tox and No-Tox samples and assessed over time. The figure illustrates data from the pilot study (0 -16 weeks) and data from the present study (after 40 weeks). Time was calculated as date of sample collection at post-mortem examination to date of DNA extraction.*

As indicated above, DNA concentration for grey-top tube samples unexpectedly increased over time. To explore this further, and in an attempt to explain this trend, the Ct values for the IPC of each red and grey-top sample were assessed to compare the possible presence of PCR inhibitors in these samples, and if these changed over time. If PCR inhibitors decreased with time, this would manifest as an apparent increase in DNA concentration measured using a PCR-based method. However, contrary to this hypothesis, the results showed an increase in the IPC Ct values for grey-top tube samples. This meant that PCR inhibitors actually increased over time and could not be the reason for the apparent increase in DNA concentration over time observed in the DNA from grey-top tubes (Figure 3.3). The IPC Ct value for red-top tubes were as expected.

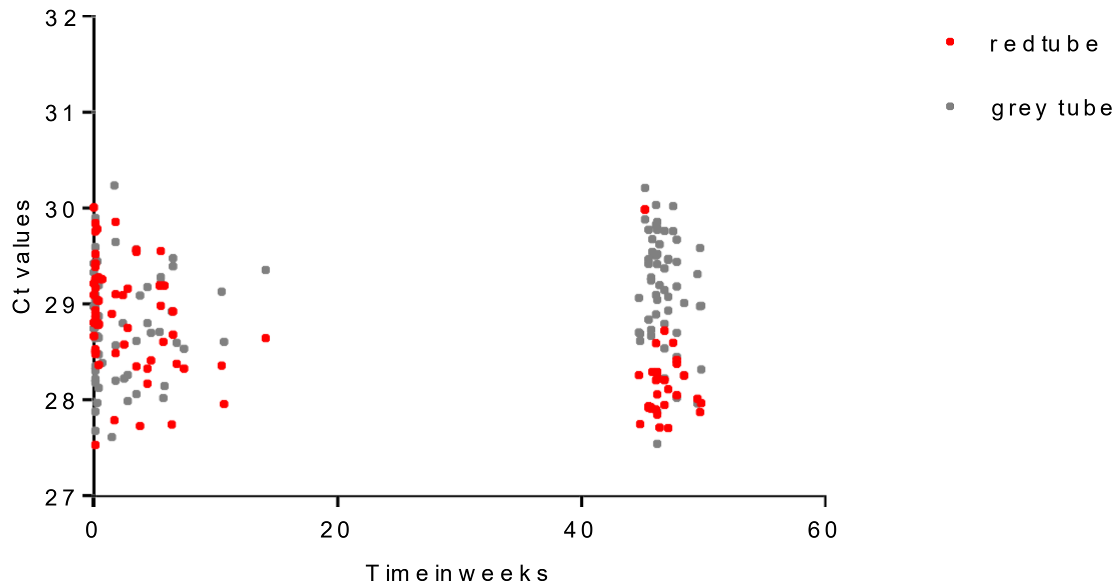


Figure 3.3: Ct values for the IPC over time in weeks. *Ct values for the IPC for red and grey-top top samples were assessed to determine the amount of PCR inhibitors present in the sample over time. Time in weeks from 0 to approximately 16 weeks represents the pilot study and data after 40 weeks represents the present follow-up study.*

3.3. Assessment of DNA quality

3.3.1. DNA degradation

The Quantifiler® Trio DNA Quantification kit (Thermo Fisher Scientific, Massachusetts, USA) measured the concentration of large and small autosomal DNA targets. DI was then calculated for all samples after 48 weeks and was used as a measure of DNA quality (Figure 3.4). P-No-Tox samples showed the highest DI (median = 1.2), followed by R-No-Tox and R-Tox with median values of 1.10 and 1.07 respectively. Grey-top samples showed the lowest DI with 0.8 for G-No-Tox and G-Tox with 0.9. There was no significant difference between G-No-Tox vs G-Tox ($p = 0.086$) as well as between R-No-Tox vs R-Tox ($p = 0.14$). Although the purple-top tubes had the highest DI, it should be noted that it was not significantly higher than the other samples, nor did it compromise the ability of molecular analysis in those samples.

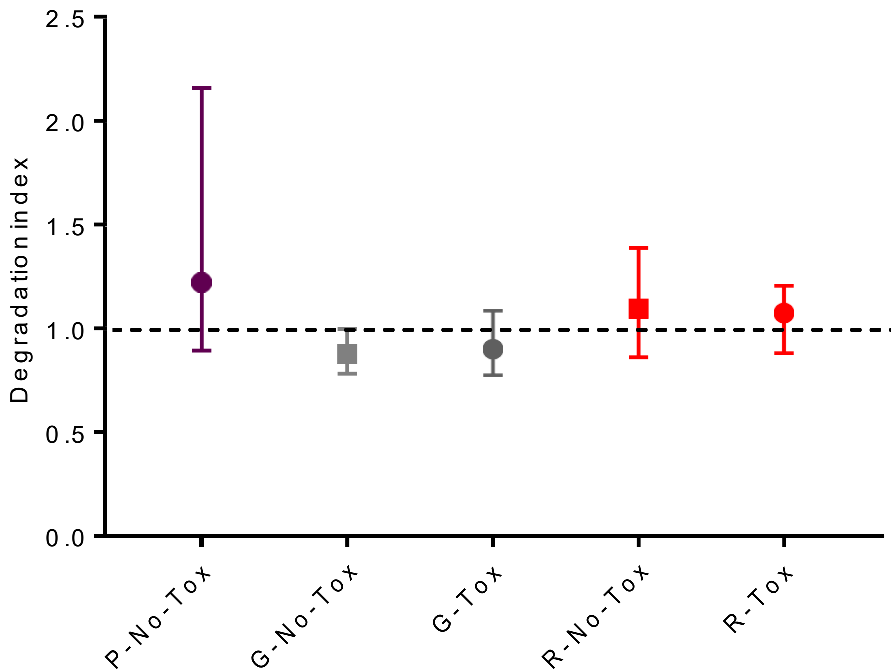


Figure 3.4: Assessment of DNA quality by measure of degradation index after 48 weeks. *Degradation index was calculated by dividing concentration of small autosomal and large autosomal DNA targets, for each sample type. Quantification of the autosomal targets was performed with the Quantifiler® Trio DNA Quantification Kit (Thermo Fisher Scientific, Massachusetts, USA).*

Degradation was assessed 48 weeks after sample collection at autopsy and compared to data from the pilot study, for red and grey-top tubes, and an increase in DI was observed for both sample types over time (Figure 3.5). In this study, 34/60 (57%) red-top and 22/60 (37%) grey-top samples had a DI between 1 and 5, indicating that DNA in these samples were slightly more degraded compared to the pilot study. There was also a single sample from a red-top tube with a much higher DI of 9.9, indicating that this sample was indeed degraded. Other than this sample, DNA degradation was not a concern after 48 weeks.

Overall, there was a positive correlation between time and DI, for grey-top samples ($r = 0.01$, $p = 0.85$) and red-top samples ($r = 0.42$, $p < 0.001$). A linear regression model found that 13% of the variance in DI could be explained by the tube type (additives in the tube) and time between blood collection at autopsy and DNA extraction ($p < 0.001$). The following equation was obtained to model DI based on these two variables:

$$DI = 0.018\ (\text{tube type}) - 0.003\ (\text{weeks between blood collection at autopsy and DNA extraction}) + 1.13$$

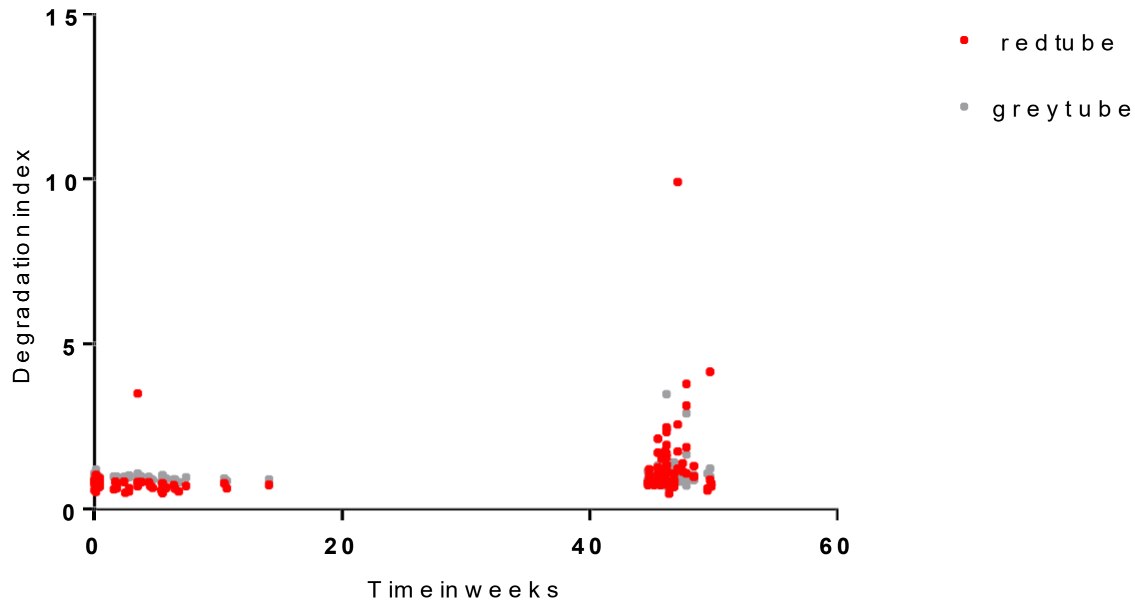


Figure 3.5: Degradation index over time. *DNA degradation for red and grey-top vacutainers (Tox and No-Tox) was combined and assessed at 16 weeks and after 48 weeks (pilot and follow-up study respectively). Time was calculated from date of sample collection at autopsy to date of DNA extraction.*

3.3.2. DNA purity

DNA purity was assessed by measuring absorbance at 260, 230 and 280 nm. Purity ratios were then calculated from the three wavelengths *i.e.* $A_{260/280}$ and $A_{260/230}$. In this follow-up study, the highest purity ratio for $A_{260/280}$ was observed with the G-Tox samples (median = 1.74), thus indicating the best purity. There was no significant difference between Tox and No-Tox samples for grey-top samples ($p = 0.15$). $A_{260/280}$ for red-top (median = 1.5) and purple-top samples (median = 1.5) were also below the threshold of 1.8, as indicated by dotted line in Figure. 3.6. Red and grey-top samples showed a decrease in $A_{260/280}$ over time, which indicated the increasing presence of protein contaminants, or the decreasing concentration of DNA; the latter which is perhaps more likely (particularly with red-top tubes). This correlation was significant ($r = -0.55$, $p < 0.001$ and $r = -0.31$, $p < 0.001$), respectively. A linear regression showed that 13.2% of the variance in $A_{260/280}$ could be explained by tube type and time between blood collection at autopsy and DNA extraction ($p < 0.001$). The overall equation was as follows:

$$A_{260/280} = 0.04 \text{ (tube type)} + 0.0017 \text{ (weeks between blood collection at autopsy and DNA extraction)} + 0.73.$$

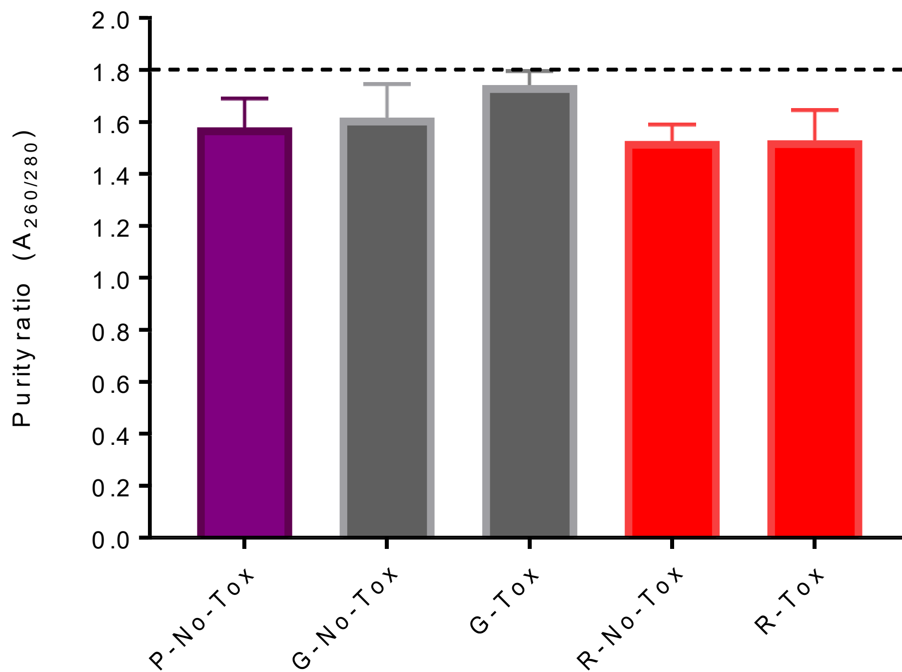


Figure 3.6: DNA purity ratio $A_{260/280}$ for blood samples measured after 48 weeks. *Following DNA extraction, samples were assessed for purity by measuring absorbance at 260 and 280 nm using the Nanodrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA).*

The highest $A_{260/230}$ purity ratio was observed with grey-top samples (median 1.2 for G-No-Tox followed by 1.1 for G-Tox). Red-top samples showed the lowest $A_{260/230}$ ratios with median values of 0.72 (R-No-Tox) and 0.61 (R-Tox). There was no significant difference between Tox and No-Tox samples for grey-top samples ($p = 0.5$), but for red-top samples there was a significant difference ($p = 0.06$). All samples fell below the threshold of 2, as shown by the dotted line (Figure 3.7). The decrease in $A_{260/230}$ for red and grey-top samples showed a significant correlation ($r = -0.61$, $p < 0.001$ and $r = -0.31$, $p < 0.001$), respectively. A linear regression analysis modelled $A_{260/230}$ based on time between blood collection at autopsy and DNA extraction and tube type and showed that the variables accounted for 38.9% of the variation seen in $A_{260/230}$. The overall equation was as follows:

$$A_{260/230} = 0.020 \text{ (tube type)} + 0.0059 \text{ (weeks between blood collection at autopsy and DNA extraction)} + 0.74$$

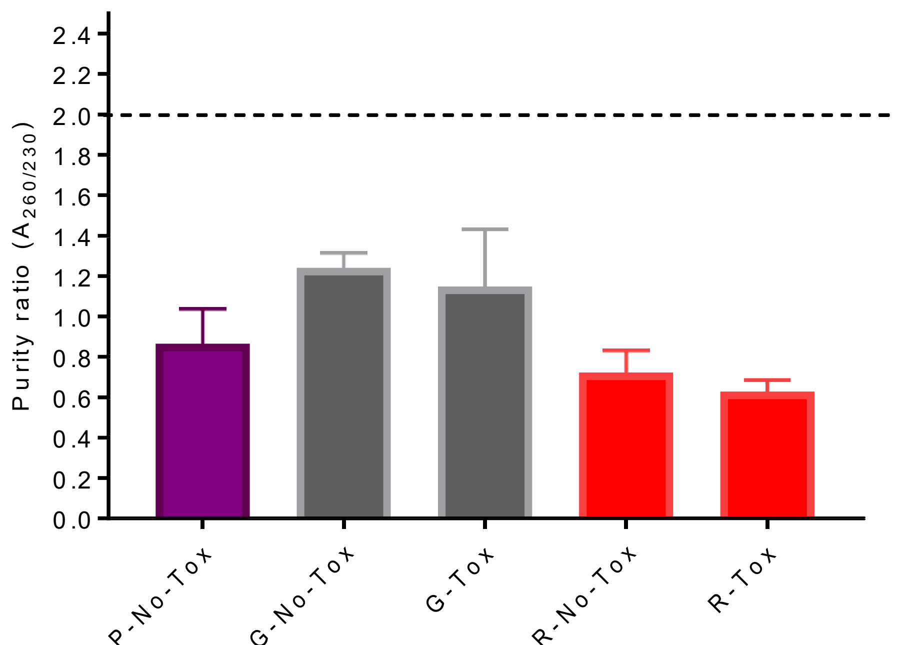


Figure 3.7: DNA Purity ratio $A_{260/230}$ after 48 weeks (follow-up study). *DNA purity was determined by Nanodrop™ 2000 (Thermo Fisher Scientific, Massachusetts, USA). Absorbance was measured at 230 and 260 nm and purity ratios calculated thereafter.*

3.3.3. Sanger sequencing

Lastly, DNA suitability for toxicogenetic analysis was assessed by Sanger sequencing. Exons 1, 4, 8 and 9 were amplified by PCR, after which agarose gel electrophoresis was performed to confirm amplification. The PCR product was used as a template for secondary PCR in red-top tube samples due to low concentrations of DNA obtained from those samples. DNA sequencing EPGs were then compared in terms of background noise and ability to accurately distinguish SNPs. The expected amplicon sizes were as follows: exon 1.1: 954 bp; exon 1.3: 314 bp; exon 4: 266 bp; exon 8: 534 bp and exon 9: 520 bp. All the exons eventually amplified successfully (Figure 3.8).

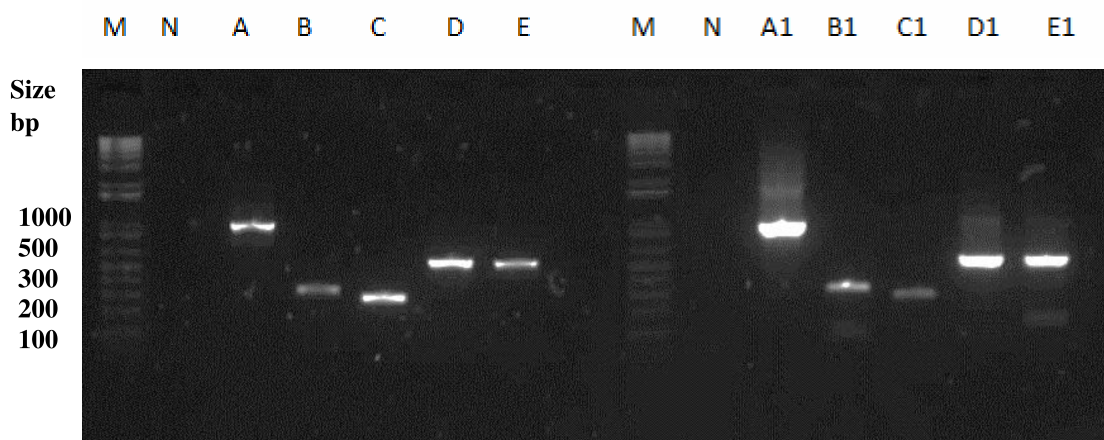


Figure 3.8: Agarose gel showing PCR amplification of *CYP2D6* targets. *Amplification was performed for regions 1.1, 1.3, 4, 8 and 9 for red and grey Tox samples. From left, well M: 1Kb Plus DNA ladder (Thermo Fisher Scientific, Massachusetts, USA), N: no template control, A-E: targets 1.1, 1.3, 4, 8, 9 respectively for grey-top samples. A1-E1 represented the same targets for red-top samples. Electrophoresis was performed at 80 V, 400 mA for 60 minutes.*

DNA sequence alignments for red and grey-top samples were compared following Sanger sequencing. The overall results, in this follow-up study, showed that grey-top samples produced EPGs that were of better quality than red-top samples, in terms of background noise and chemical artefacts. EPGs were of poor quality for exon 1.1 (954 bp) for the red-top tube, while for the grey-top it was of fair quality. EPGs for exon 9 (520 bp), for both red and grey-top tubes were unusable; while EPGs were of fair quality for exon 1.3 (314 bp) and 8 (534 bp) for the red-top samples. Exon 4 (266 bp) was the only region which produced good quality EPGs for both red and grey-top samples and showed the same variations as observed during the pilot study. Overall data was good for the grey-top tubes in three instances, while it was poor, fair or unusable for red-top samples. As expected, there were no new artefacts or variations observed in any of the EPGs of good quality. Table 3.1 below summarises the quality of the Sanger sequencing results, alongside those results from the pilot study.

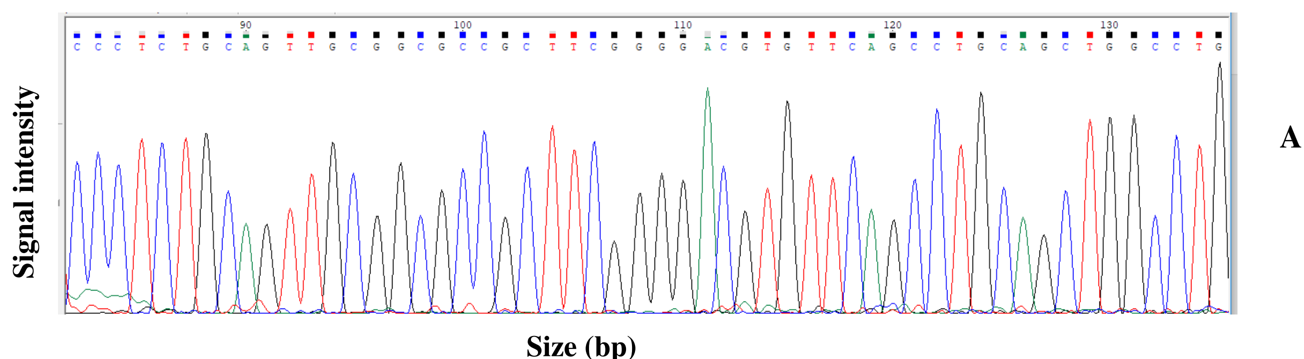
Table 3.1: Comparison of EPG quality in 2017 and 2018

Region	Size (bp)	Red-top tube		Grey-top tube	
		2017	**2018**	**2017**	**2018**
4 F	266	Good	Good	Good	Good
4 R		Good	Good	Good	Good
1.3 F	314	Good	Poor	Good	Good
1.3 R		Good	Good	Poor	Good
9 F	520	Fair	Unusable	Poor	Unusable
9 R		Fair	Unusable	Fair	Unusable
8 R	534	Good	Fair	Good	Good
1.1 F	954	Good	Unusable	Good	Poor
1.1 R		Fair	Unusable	Fair	Good

Table 3.1: EPG quality at 16 weeks (2017) and 48 weeks (2018) after sample collection at autopsy. *After quantification, DNA suitability for toxicogenetic analysis was assessed using Sanger sequencing. Results from the pilot study were then compared to results obtained in the follow-up study.*

In summary, red-top samples had 4 EGPs which were unusable and one 1 poor, compared to 2 unusable and 1 poor in grey. But overall, out of the 5 regions, 2 were unusable for red, and only 1 unusable for grey.

Figure 3.8 below shows exon 1.3 for grey and red-top samples to illustrate good and poor - quality EPGs (respectively). The rest of the DNA sequences are shown in appendix C.



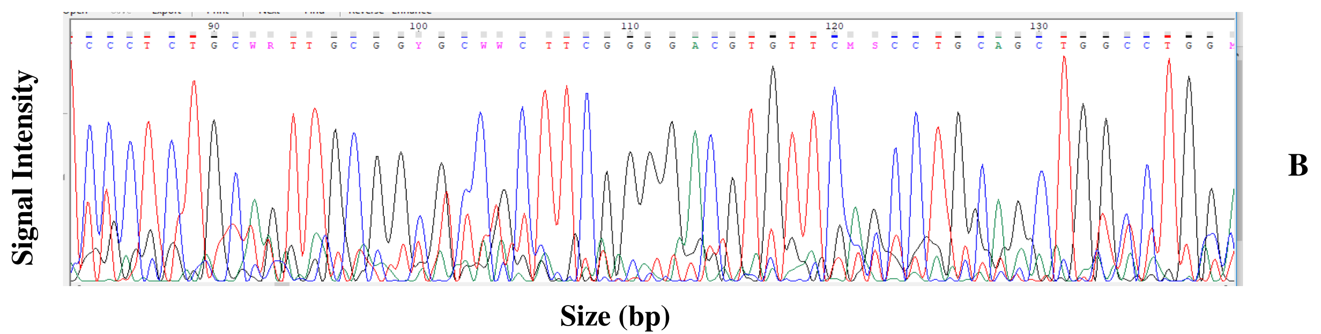


Figure 3.9: A portion of DNA sequence for exon 1.3 for (A) grey- and (B) red-top samples. *Following DNA extraction in the follow-up study, five targets of CYP2D6 were amplified using case 22 red and grey-top samples that had undergone toxicological analysis. The samples were then sent to the Central DNA Sequencing Facility at the University of Stellenbosch for sequencing. A represents grey-top while B represents red-top samples.*

CHAPTER 4: DISCUSSION

The aim of this research was to determine if blood samples collected into NaF/$C_2K_2O_4$ and plain tubes (grey and red-top respectively) for toxicological analysis could be used for subsequent genetic analyses, should that be warranted as the next step of ancillary investigations to determine CoD and/or MoD. In this phase of the longitudinal study, the focus was DNA suitability for genetic analysis 48 weeks after sample collection, and to compare results to those obtained initially. This study was situated in a larger study, which aims to investigate the potential and future use of toxicogenetic analysis in local post-mortem examinations. This was motivated by the genetic analyses of DMEs related to ADRs and death, which have shown value in aiding CoD and/or MoD in other countries (Levo *et al*., 2003). It is therefore important in our context to determine if the blood samples already collected for toxicological analyses would be suitable for subsequent genetic analysis; as opposed to collecting an additional sample for DNA analysis in every case – and it only being needed in the minority of cases.

4.1. DNA concentration

DNA concentration must be estimated accurately before any downstream analyses such as DNA profiling or sequencing (Yu *et al*., 2017). DNA quantification by Nanodrop™ 2000 spectrophotometry (Thermo Fisher Scientific, Massachusetts, USA) was performed to determine if there were samples that required dilution prior to qPCR. qPCR is a very sensitive and specific DNA quantification method, which specifically measures the amount of amplifiable DNA (Köchl, Niederstätter & Parson, 2005). Accurate quantification of DNA in a forensic sample makes it possible to adjust its concentration for downstream analysis such as for genotyping or sequencing (Nielsen *et al*., 2008).

The decrease in DNA concentration observed for red (Figure 3.2) and purple-top tubes (Appendix B), after 48 weeks, could possibly been have influenced by post-mortem cell death. Cell death occurs via a process known as necrosis, which is characterised by swelling of cytoplasmic organelles, increased cell volume and condensation of chromatin (Alaeddini, Walsh & Abbas, 2010). This is then followed by the rupture of membranes and spilling of lysosomal enzymes into cellular compartments. These effects have been observed in stored

whole blood (Alaeddini, Walsh & Abbas, 2010), suggesting that this may have been a plausible mechanism for the DNA loss observed in this study.

Independent of whether blood samples had undergone toxicological analysis or not, the concentration of DNA isolated from blood that was stored in grey-top tubes was the highest 48 weeks later. This was in comparison to DNA concentration from purple and red-top tube samples at the same time point (Figure 3.1). There were significant differences in DNA concentration between each tube type; thus, it was postulated that the additives in the different tubes played a role in altering DNA concentration.

Purple-top samples were stored in EDTA, which was recommended as the anticoagulant of choice as it inhibited DNAse activity and is reported to not alter quantity of DNA if stored appropriately, such as in the freezer for long-term (Kotikalapudi & Patel, 2015). Several guidelines for optimal DNA storage for DNA extraction have been reported, to allow for genetic studies to be conducted over a long period of time (Gomma & Fox, 2001). DNA was reported to be more stable under long-term freezer storage and storage in buffy coats, which are aliquots of white blood cells (Mychaleckyj *et al*., 2011). Another study also investigated the effect of temperature and storage time on blood samples stored in EDTA. The results showed that extracted DNA was of high quality after storage at 4 ºC for a maximum of one month (Nederhand *et al.*, 2003). Although EDTA tubes were used for the controls in this study, these samples were stored at 4 ºC and not frozen for one year. This was to expose the controls to the same conditions as those experienced by the other tubes so as to minimise variability. However, the prolonged storage of samples in purple-top tubes under conditions which are perhaps not the most ideal, could therefore explain the decreasing DNA concentrations, as well as DNA concentrations that were lower than the grey-top tubes.

The red-top tubes contained no additives, suggesting that it had no added protective measure against DNA damage. There has been research investigating the effect of storing whole blood without preservatives for DNA fingerprinting. In a study performed over a period of eight weeks, blood was stored in tubes with either EDTA, heparin or without the anticoagulants at room temperature, 4 ºC, -20 ºC and -70 ºC (Bomjen *et al.*, 1996). The results showed that DNA yield was better in blood samples stored with anticoagulants (EDTA or heparin) and at colder temperatures (Bomjen *et al*., 1996). It has been reported that prolonged storage of whole blood causes lysis of white blood cells (WBC) resulting in DNA loss during the WBC harvesting step

of DNA extraction (Nederhand *et al.*, 2003). This process seemed to be slowed by the presence of additives, EDTA or heparin (Bomjen *et al.*, 1996). Hence the significant decrease in DNA quantity observed in red-top tubes in this study (p = 0.01) could be attributed to the lack of additives in the tube.

Grey-top tubes, containing NaF preservative and $C_2K_2O_4$ anticoagulant exhibited the highest DNA concentrations (for both Tox and No-Tox samples) in comparison to red and purple-top samples. There have been studies to show effect of fluoride on DNA degradation, however no studies could be found to show effect the of fluoride on DNA concentration. In this study grey-top tubes had the highest DNA quantity, compared to standard purple-top tubes after 48 weeks of storage. Regression analysis showed that time and tube type could only explain 14% of the variation in concentration, where time accounted for the majority (9.6%) of the variance. This therefore suggested that other factors were at play. This then prompted the assessment of Ct values of the IPC over time (between 16 and 48 weeks) to determine if PCR inhibitors could explain the observed results for grey-top tubes.

It was thought that the apparent increase in DNA concentration in grey-top tubes may be due to a decrease in PCR inhibitors. It was speculated that in 2017, there were inhibitors present in the blood samples, which therefore underestimated DNA concentration, and with time the inhibitors may have decreased, showing an apparent increase in DNA concentration. However, this was not the case, as the analysis of the Ct values of the IPC showed an increase of PCR inhibitors over the 48-week period. Although it was not the primary objective Nanodrop™ spectrophotometry, DNA concentration was measured for all samples, which also confirmed the overall increase in DNA concentration for grey-top samples (data not shown). This provided further evidence that the apparent increase of DNA concentration as measured by qPCR was indeed not due to a decrease in PCR inhibitors.

These results then point to the possibility of more DNA being extracted from the vials in this study compared to the pilot study. This may have been due to the chemicals present in the grey-top tubes which may have interacted with reagents during the DNA extraction process differently over time. To investigate this hypothesis and the possible chemical interactions was beyond the scope of this minor dissertation, but warrants further investigation to better understand the trends observed in this study.

Lastly, inter-user variability between the two studies may have been a reason for the apparent increase of DNA concentration in samples stored in grey-top tubes. It is possible that there were slight differences in the DNA extraction protocol or even the preparation of standards or sample dilution for qPCR, resulting in the results observed. For example, more DNA could have been extracted during the present study, as a result of inverting the blood collection tubes more. If this was the case, a systematic trend across all tube types would be expected, but the increase in DNA concentration was only (and consistently) observed in grey-top tubes. However, user variability cannot be excluded totally as a contributory factor in the apparent increment in DNA concentration for grey-top tube samples.

4.2. Assessment of DNA quality by purity ratios

Analysis of both purity ratios showed decreases in DNA purity over time for all tube types (Figure 3.6 and Figure 3.7), with the decrease in $A_{260/230}$ being greater than the decrease in the $A_{260/280}$ ratio. A regression model for $A_{260/230}$ based on time and tube type showed that 38.9% of the variance in this ratio could be explained by tube type and time and this percentage variance was the highest for all variables investigated.

While both purity ratios for all samples were below the accepted thresholds of 1.8 and 2, samples from the grey-top tubes were the purest compared to the other tube types. The additives in the various tubes must also be considered within the context of spectrophotometry interpretation. For example, EDTA and $C_2K_2O_4$ absorb UV at 230 nm (Bhattacharyya & Kundu, 1971; Soga & Ross, 1999) which may have contributed to the absorbance at 230 nm measured in this study, particularly if it was not sufficiently removed during the DNA extraction process. Although EDTA alone does not absorb UV light at 230 nm, the interaction with iron (Fe^{2+}) found in blood does (Bhattacharyya & Kundu, 1971).

As such, the $A_{260/230}$ purity ratio would be representative of not only the traditional chaotropic salts and carbohydrates known to absorb light at 230 nm and to be involved in the sample and/or DNA extraction process, but also from the EDTA or $C_2K_2O_4$ from the purple-top and grey-top tubes, respectively. NaF has a maximum absorbance at 500 nm (Farajzadeh, 2004), and thus was unlikely to have affected the purity ratios measured in this study. However, it still may have decreased the overall purity of the sample but was not measured using the spectrophotometry method. The use of spectrophotometry therefore was a limitation of measuring sample purity in this study.

4.3. Assessment of DNA quality by degradation index

Analysis of degraded DNA is a common challenge in forensics due to the wide range and quality of samples encountered. The Quantifiler® Trio DNA Quantification kit (Thermo Fisher Scientific, Massachusetts, USA) measures the quantity of small and large autosomal targets, the ratio of which is used to assess degradation (Köchl *et al*., 2005). Samples are not degraded if the small and large fragments amplify equally. However, if the smaller fragments amplify better than the large fragment, then a DI > 1 is observed, meaning there is a greater degradation of the DNA. Research has shown that when DI > 4, challenges may be encountered in downstream assays where amplification of fragments larger than 300 bp is necessary (Köchl *et al*., 2005).

The average DI for all samples in the pilot study (*i.e.* approximately 16 weeks from sample collection at autopsy) were below 1, suggesting that DNA was intact and not degraded (Figure 3.5). Measurement of DNA extracted after 48 weeks showed that DI increased slightly to a median of 1.2, which was the highest for purple-top tubes. Red-top tubes had a median DI of approximately 1, whilst the grey top-tubes recorded the lowest index of 0.8. Although there was a significant increase in DI for red-top ($p < 0.001$) and grey-top tubes ($p = 0.02$) (Figure 3.5), the DI surrounding 1 meant that DNA was generally not degraded for any sample type after 48 weeks. The exception was one outlier, for red-top tubes, with a DI of 9.9 and was probably due to the drugs found in the system.

There have been studies showing that NaF induces DNA damage in rat kidneys (Ning *et al*., 2009), however there was limited information on the mechanism of this fluoride-induced cell death. One option put forward was that fluoride led to an increase in Ca^{2+} in different cell types and that this increase was proposed to induce apoptosis (Trump & Berezesky, 1992). In another study, fluoride was shown to induce DNA damage in human carcinoma cells (Verma, Daoud & Pathak, 2017). These data suggest that fluoride does have an influence on DNA damage or degradation thereof, which means more work is required to investigate the effect of fluoride on human DNA. There was no data to show effect of $C_2K_2O_4$ on DNA as the two ($NaF/C_2K_2O_4$) together might change the way NaF interacts with DNA.

Although purple-top tubes are the standard collection tubes for genetic analyses, DNA degradation was highest in these tubes, although not significantly higher than those from red

and grey-top tubes. This was likely attributed to the effect of storing blood at 4 ºC whereby DNA quality has shown to decrease at this temperature over time, even when stored in Tris-EDTA (TE) buffer (Richardson *et al*., 2006; Lahiri and Schnabel, 1993).

A regression model showed that storage time and tube type could explain 14% of variations seen in DI. This again suggests that other factors are at play, such as age, sex and post-mortem interval. The assessment of these variables again fell out of the scope of this investigation, but will be considered in future work.

4.4. Sanger sequencing

In this study, post-mortem blood samples collected in grey-top and red-top tubes were analysed for suitability to perform toxicogenetic analyses. Toxicogenetics focuses on the genetic variations in genes responsible for drug metabolism. CYP2D6 is involved in the metabolism of many toxicologically important drugs and genotyping for this enzyme resulted in polymorphic distributions in various populations examined (Druid *et al*., 1999). *CYP2D6* genotyping is considered a valid technique to determine metabolic status (Sistonen *et al.*, 2005). In this study, genotyping would assist in determining the metabolic status of the deceased and contextualise as to whether any drugs detected may have been affected by these metabolic alterations.

The purpose of this analysis in this study was to amplify DNA in one selected case from the cohort, specifically the target regions where SNPs were observed previously, and compare the results from this analysis to those obtained in the pilot study (Vuko, 2017). The pilot study showed that good sequencing data was consistently obtained from the red-top tube samples, whereas many artefacts were observed in EPGs from DNA from the grey-top tubes. The purpose of repeating the sequencing analysis in this study was two-fold: first, to determine whether the assay could be carried out on samples with lower concentrations and increased degradation of DNA (red-top tubes) and (ii) if the assay would still produce reliable results approximately one year later. This was important considering the long delays in receiving toxicological results in the local context and therefore possibility of initiating a toxicogenetics analysis. Only after toxicology results are returned, would genetic analysis be requested, which could be some time (e.g. months to years) later from the FCLs.

Quantity and quality assessment showed a significant decrease in concentration and purity ratios and an increase in DI for red-top tubes, which was consistent with the challenges experienced in analysing these samples using PCR and sequencing methods. The PCR assay was re-optimised for red-top tubes due to their significantly lower concentration as compared to the pilot study (Vuko, 2017) (Figure 3.2). Upon initial performance of the original assay, samples from red-top tubes showed faint bands on the agarose gel (Appendix D) and during sequencing, had a very low signal to noise ratio. Amplification was better for the smaller fragments. To overcome this challenge, a secondary PCR was carried out for larger exons, with altered PCR conditions, as described in Chapter 2.

Despite these improvements, EPGs from red-top samples were still of slightly poorer quality compared to matched exons from grey-top tubes. The poorer performance of the red-top tube samples was attributed to the very low concentration of DNA observed 48 weeks after sample collection (median = 0.09 ng/µl), compared to 16 weeks after sample collection (median = 17.4 ng/µl). While red-top tube samples were more degraded over time in general, the median DI of this sample of interest was 1.7, which indicated only slight degradation, and this was probably not a factor for poorer sequencing. However, if blood samples from red-top samples were the only samples available, an attempt could be made to perform toxicogenetics analysis, but the target amplicons need to be kept small and a DNA polymerase which is sensitive to low copy number DNA must be used. It is also anticipated that with increased age of the samples, issues with Sanger sequencing may become more complex, and the procedure may require further optimisation. This might include the adjusting of magnesium (Mg^{2+}), a cofactor of *Taq* polymerase, and/or the adjustment of pH of the PCR buffer.

Assessment of grey-top tube samples in the pilot study showed EPGs with chemical artefacts (Vuko, 2017), which was not the case at 48 weeks. EPGs for two regions (exons 4 and 8) remained the same good quality over time, and the EPGs for exon 1.3 improved in quality over time (*i.e.* poor to good). The improvement may have been due the higher concentrations of DNA obtained from the samples in this study, or perhaps due a decrease of chemical interactions from the additives in the grey-top tubes (which was not investigated further in this study). The sequencing of exon 9 was problematic in both the red and grey-top tube samples, and was of poor or fair quality in the pilot study too, suggesting that this result might be region-specific, as opposed to tube-type related. A closer analysis of the primers and possible variants within the primer binding regions need to be assessed in this case example in the future. The

relatively good quality sequencing results from this study are in accordance with a previous study by Druid et al. 1999, where *CYP2D6* genotyping was successful on femoral post-mortem whole blood, where EDTA, heparin and NaF was added (Druid *et al*., 1999).

The final region sequenced in this study was the first portion of exon 1 (named exon 1.1) and the EPG from the grey-top tube decreased in quality compared to that obtained in the pilot study. The reason for this observation was that slightly different primer sets were utilised in this study which resulted in a larger fragment of DNA being amplified here. During the pilot study, primer set for exon 1.1 targeted a fragment of 439 bp. However, subsequent to this, genetic variations were found in the primer binding region for the reverse primer, resulting in failed amplification in some samples. Therefore, a new reverse primer was designed, but to mitigate non-specific binding, it resulted in an amplicon of 954 bp (Vincent, 2017). The decrease in EPG quality for this fragment was thus attributed to the larger amplicon size and not type tube.

It is not always easy to amplify short target sequences with *CYP2D6* due to presence of pseudogenes (Gaedigk, 2013) and non-specific binding. Next generation sequencing (NGS) thus might be useful in this regard as it reads a large number of short fragments in a high-throughput and parallel manner, with a read length of about 35-500 bp (Zhang *et al.*, 2011). Since NGS technology is rapidly increasing the speed of throughput capacities for DNA sequencing, it is also becoming more affordable (Schuster, 2008).

While overall sequencing of DNA from grey-top tubes was better than that from red-top tubes in this study, such a conclusion cannot be made based on such small sample sizes. It would be important to sequence all nine exons of *CYP2D6* and on a statistically significant number of cases to get a realistic indication of which tube type(s) would be suited for this analysis. This study has nevertheless provided important insight into the unexpected results if improved results from grey-top tubes with time, as well as some of the challenges encountered when dealing with low copy number DNA in this context.

4.5. Challenges with toxicogenetics interpretation

The challenges in toxicogenetics are not only technical, but the genetic information it provides needs to be interpreted within the case context. Firstly, the result must be integrated with those from forensic toxicology and forensic pathology (Sajantila *et al.*, 2010). This means that knowledge of pathophysiological conditions, concentration of drugs and their relative metabolites at the time of death, as well genetics results must be combined for such investigations (Sajantila *et al.*, 2010). Additionally, fundamental research must still be conducted before toxicogenetics results can be routinely used as evidence in court, so that there is enough knowledge to render reliable interpretations.

Toxicogenetic studies to date have established strong associations between genotype and phenotype for a limited number of cases (Koski *et al.*, 2006). Research stated that genotype does not always translate to phenotype, but rather suggest an alternative explanation for CoD and/or MoD (Sajantila *et al.*, 2010). Another issue with toxicogenetic studies is that a change in amino acid sequence of an enzyme does not directly indicate a change in phenotype. In addition variants around the coding regions of the DME genes can affect metabolism without having an effect on structure of the protein (Sajantila *et al.*, 2010). This complicates interpretation of medico-legal genetics and as such, caution must be exercised.

Some SNPs and/or haplotypes reported as prevalent in other populations remain to be fully investigated and understood in African populations as contradicting results have been found (Matimba *et al*., 2009). *CYP2C9*2* and *CYP2C9*3* for example, identified as rare alleles in African Americans were not found in the African population (Matimba *et al.*, 2009). Although the data were reported for *CYP2C9*, the same can be inferred for *CYP2D6* as they are similar in functionality. Llerena *et al* (2014) reported an uneven distribution of *CYP2D6* studies around the world, with Africa highly underrepresented (LLerena *et al*., 2014). The lack of frequency data and functional studies regarding variations observed, often makes it difficult to predict what the variants mean for the deceased individual. Although this follow-up study did not assign haplotypes, this was done in the pilot study and could potentially be done in further studies. This will add to the currently available data on SNPs/haplotypes regarding South African population or Africa as a whole.

The presence of pseudogenes also causes problems with *CYP2D6* genotyping (Endrizzi *et al*., 2002). The *CYP2D* locus contains two pseudogenes *CYP2D7* and *CYP2D8* which are closely related and located to *CYP2D*6 (Gaedigk, 2013). This in turn presents challenges with primer design as well as with full gene sequencing and genotype interpretation (Yang *et al*., 2017). *CYP2D6* is also susceptible to allele drop-out due to the fact that it is highly polymorphic (Scantamburlo *et al*., 2017). This is especially problematic when a phenotype is predicted from a genotype, as this might lead to phenotype misclassification (Scantamburlo *et al.*, 2017). The large number of allelic variants as well as presence of structural and copy number variation has presented challenges with *CYP2D6* genotype analysis (Gaedigk, 2013).

4.6. Limitations, considerations and future work

There were several limitations to this study, the first being that the selection criteria of cases excluded those with signs of decomposition. Decomposition is not uncommon in forensics, but the data from the current study does not provide insight into suitability of blood samples from these cases. However, the approach used in this study was chosen to first establish baseline data, which must be followed up with testing on more representative cases in the future.

A current limitation in this study was the gap in storage time between analyses. Currently there are only two-time points were data has been collected, which limits the statistical analyses and modelling ability. Therefore, including more time points in the ongoing longitudinal nature of this study is warranted.

Another limitation with this study was the small sample size of 30 and it is of utmost importance to increase the number of participants going forward, to represent the large and varied population of the WC and South Africa. One of the reasons for the small sample size in this study was that this study began as a pilot project, to demonstrate a proof of concept. Since the results shown in both studies (pilot and current) are promising, the study needs to be suitably expanded to generate statistically representative and reproducible results. In this regard, the ethical concerns pertaining to the use of post-mortem samples in research need to be discussed. Sampling from deceased individuals for research purposes is an important but sensitive matter, which needs to be conducted under a strict ethical framework and be strongly justified (Heathfield *et al*., 2017). This is mainly because one must obtain informed consent from grieving family members, and such individuals are vulnerable at the time they

are often approached for consent (Heathfield *et al.*, 2017). In this larger study, informed consent was obtained from families following the strict ethical guidelines previously mentioned.

The generation and feedback of genetic results is another area of ethical concern. The question raised was whether genetic research results should be shared with family members and if so, what information should be shared. Giving genetic results to living relatives is especially important if mutations are discovered which would have implications on the remaining family members. In such cases genetic counselling becomes a necessity, to elucidate the significance of the genetic results. Returning the results of genetic testing to remaining family members means that they can also get some form of closure, after understanding what may have caused or contributed to their loved one's death (Bird, 2014).

Lagay (2000), in a study based on health effects associated with pesticide exposure, described vulnerability of a population as one of the ethical concerns in toxicogenetic studies and stated that research is a foreign concept to the majorityof the population (Lagay, 2000). Other issues raised included language barriers, which can hinder people's ability to understand the research. These are some of the considerations which must be continually taken into account in ongoing research in this field.

In this study, the variables of time and tube type accounted for 13 – 38% of variance in DNA concentration, purity and degradation. As such, it was hypothesised that other factors such as post-mortem interval (PMI), age and sex of the deceased possibly played a role in DNA quantity and quality and therefore should be investigated further. This information is available for the purposes of this study.

In the case that blood is not available for genetic analyses, DNA may be obtained from stored formalin fixed paraffin-embedded (FFPE) tissue (if available). During post-mortem examinations, human tissue samples are routinely collected and subjected to a fixation and preservation procedure for histological analysis and to allow for long-term archival storage (Hansen *et al*., 2014). Although DNA can be obtained from FFPE tissue, it was reported that the material was often degraded and cross-linked which made it difficult to amplify by PCR (Baak-Pablo *et al*., 2010). Although FFPE was not the focus of this study the sample type can be investigated for future studies.

Future work should also bear in mind the possible optimisation required for Sanger sequencing in samples with low concentrations, as well as expand this study to investigation other DMEs. This study has focused on one CYP enzyme of which there are many enzymes that can metabolise drugs and most drugs are metabolised by more than one CYP. Therefore this, coupled with multiple drugs affecting metabolism competitively becomes more complex. This might need to be taken into consideration in possible future work.

4.7. Conclusion

DNA quality and quantity were previously assessed approximately 16 weeks after sample collection and showed that no additive tube (red-top) samples were suitable for genetic analyses. In this study, at approximately 48 weeks after sample collection, the DNA quantity and quality were assessed again. DNA concentration and purity had significantly decreased for red-top tube samples, so much so that PCR needed to be re-optimised. Despite these efforts sequencing data was of poorer quality for assessing SNPs in a single case example.

In contrast, DNA from NaF/$C_2K_2O_4$ tube (grey-top) samples had an unexpected increased concentration with time, as measured by qPCR, the mechanism which remains unknown. Three out of five of the grey-top samples produced sequencing data which was suitable for assessing SNPs, which was an improvement compared to the first assessment in the pilot study.

The aim of this study was to assess the quantity and quality of DNA from blood samples, which were collected in tubes with NaF/$C_2K_2O_4$ (grey-top tubes) and tubes without preservative (red-top tubes) for toxicological screening and stored for one year. This aim has been achieved and has added to the body of knowledge concerning the pre-analytical factors associated with toxicogenetics, and in particular the considerations surrounding the quality of the genetic results.

Considering the results from both the pilot study and this follow up study, both tube types may pose challenges for toxicogenetics and further work is required to further optimise processes as well as assess DNA quantity and quality over longer periods of time. Additional longitudinal analysis therefore must be conducted on these samples to monitor trends over time, as well as these results verified in a larger sample size. These results provide important information in the understanding of DNA in blood samples stored in different tubes over time.

Forensic toxicologists are often asked to provide information concerning the role drugs/substances played in impairment or death. Furthering our understanding of toxicogenetics and individual variation in metabolism of relevant drugs (such as prescribed medicinal and recreational drugs of abuse) and associated pharmacokinetic variation may assist in strengthening these interpretations as a whole. Research in these areas may improve our understanding of the role of inter-individual metabolism and genetic variants in interpreting drug concentrations determined from post-mortem forensic toxicological analysis.

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APPENDICES

APPENDIX A: CYP2D6 primers and the exons they belong to.

Table A1: Target exons of *CYP2D6* and the corresponding primers.

Target	*CYP2D6* exon	Primer sequence (5' to 3')	Primer Direction	Length (bp)	GC content (%)	Predicted Tm (^{0}C)	Product Size (bp)
A	1.1	GCCATCATCAGCTCCCTT	Forward	18	55.6	54.9	954
		CCTATTTGAACCTTGGACGA (previously 1.2 R)	Reverse	20	45.0	52.1	
C	1.3	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	

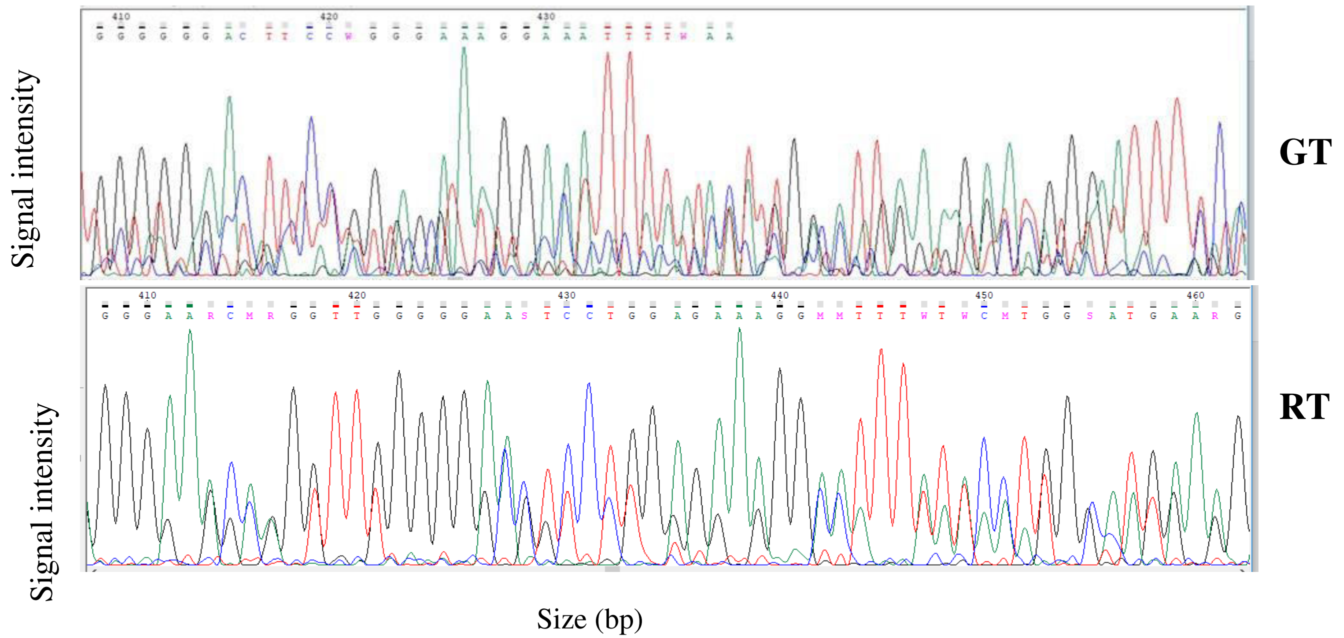
Note: Targets A and C are not full-length exons; they are parts of exon 1 of *CYP2D6*, combining to give the full length of the exon. Targets D and F are stretches of DNA incorporating exons 2 and 3, and exons 5 and 6 of *CYP2D6*, respectively.

APPENDIX B: Median values for 2017 and 2018

Tube type	**Concentration**	**DI**	$A_{260/280}$	$A_{260/230}$
R-No-Tox: 2017	25.81	0.7957	1.832	1.292
R-No-Tox: 2018	0.855	1.095	1.527	0.7225
R-Tox: 2017	9.850	0.6752	1.667	1.312
R-Tox: 2018	0.890	1.074	1.53	0.6275
G-No-Tox: 2017	20.26	0.888	1.845	1.577
G-No-Tox: 2018	37.18	0.8785	1.617	1.24
G-Tox: 2017	22.93	0.8426	1.767	1.665
G-Tox: 2018	21.46	0.900	1.742	1.1475
P-No-Tox: 2017	20.92	0.8540	1.855	1.522
P-No-Tox: 2018	6.646	1.221	1.58	0.865

APPENDIX C: Quality assessment by Sanger sequencing

Exon 1.1: Forward orientation



Reverse Orientation

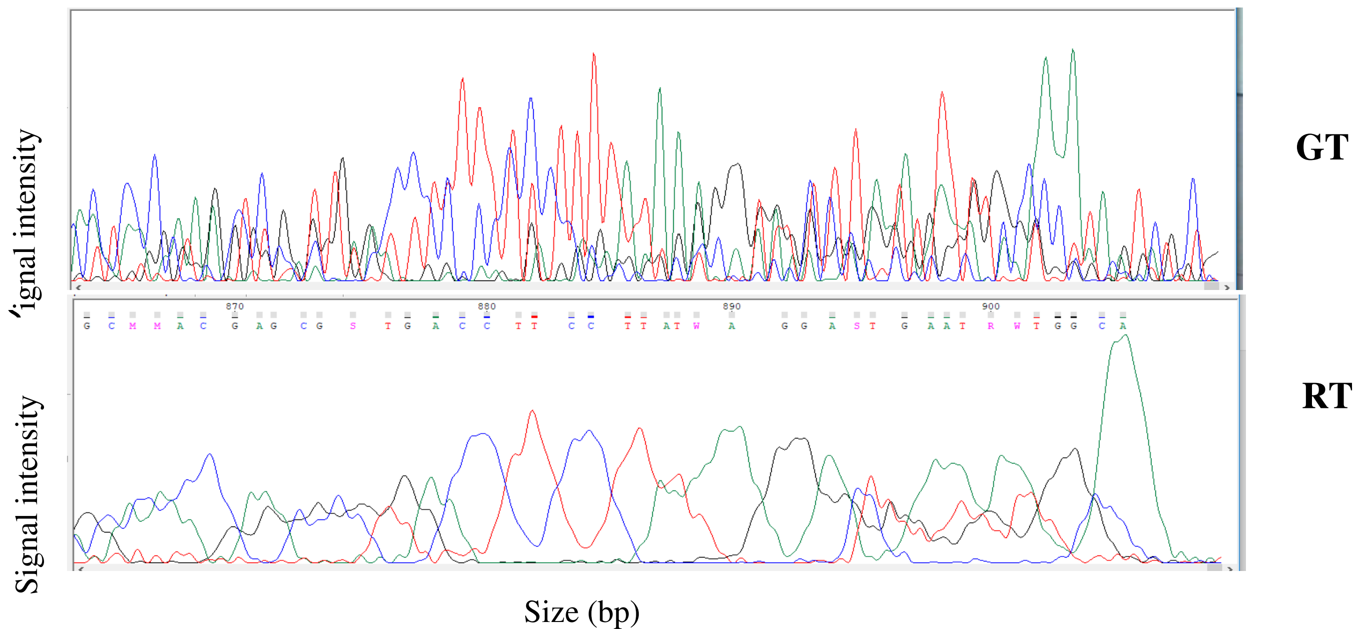


Figure C1: A portion of DNA sequence for exon 1.1 for grey and red-top samples. The figure shows the forward and reverse complements for red and grey top samples. *Following DNA extraction, five targets of CYP2D6 were amplified using red and grey samples for case 22. The samples were then sent to the Central DNA Sequencing Facility at the University of Stellenbosch for sequencing.*

Exon 1.3: Forward orientation

Signal intensity

GT

Signal intensity

RT

Size (bp)

Reverse orientation

Signal intensity

GT

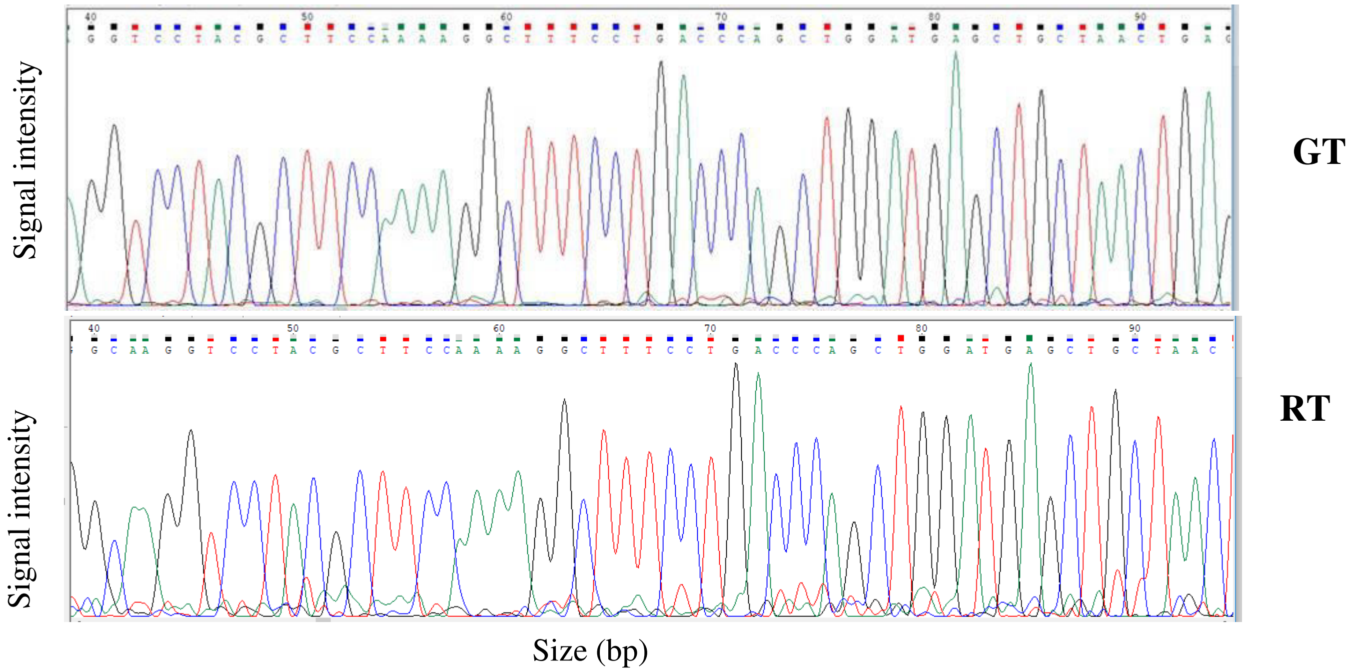
Signal intensity

RT

Size (bp)

Figure C2: A portion of DNA sequence for exon 1.3 for grey and red-top samples. The figure shows the forward and reverse complements for red and grey top samples. *Following DNA extraction, five targets of CYP2D6 were amplified using red and grey Tox samples for case 22. The samples were then sent to the Central DNA Sequencing Facility at the University of Stellenbosch for sequencing*

Exon 4: Forward orientation



Reverse orientation

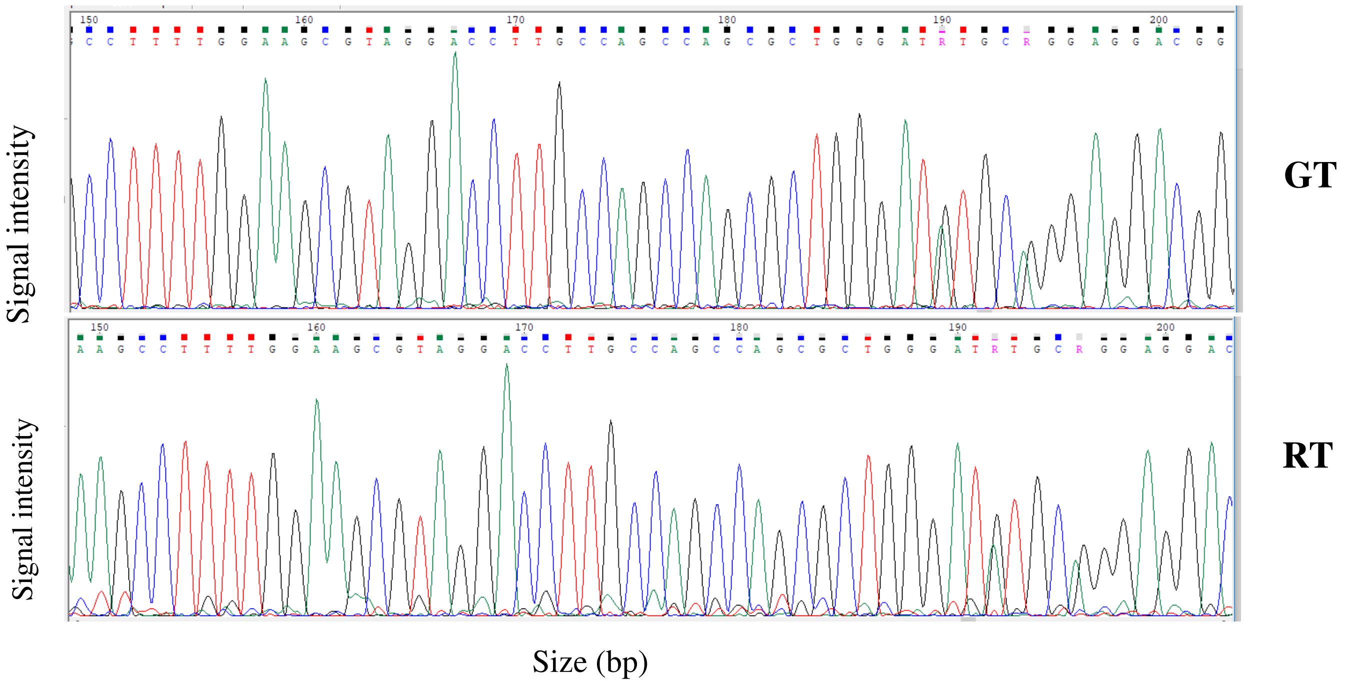


Figure C3: A portion of DNA sequence for exon 4 for grey and red-top samples. The figure shows the forward and reverse complements for red and grey top samples. *Following DNA extraction, five targets of CYP2D6 were amplified using red and grey Tox samples for case 22. The samples were then sent to the Central DNA Sequencing Facility at the University of Stellenbosch for sequencing.*

Exon 8: Reverse orientation

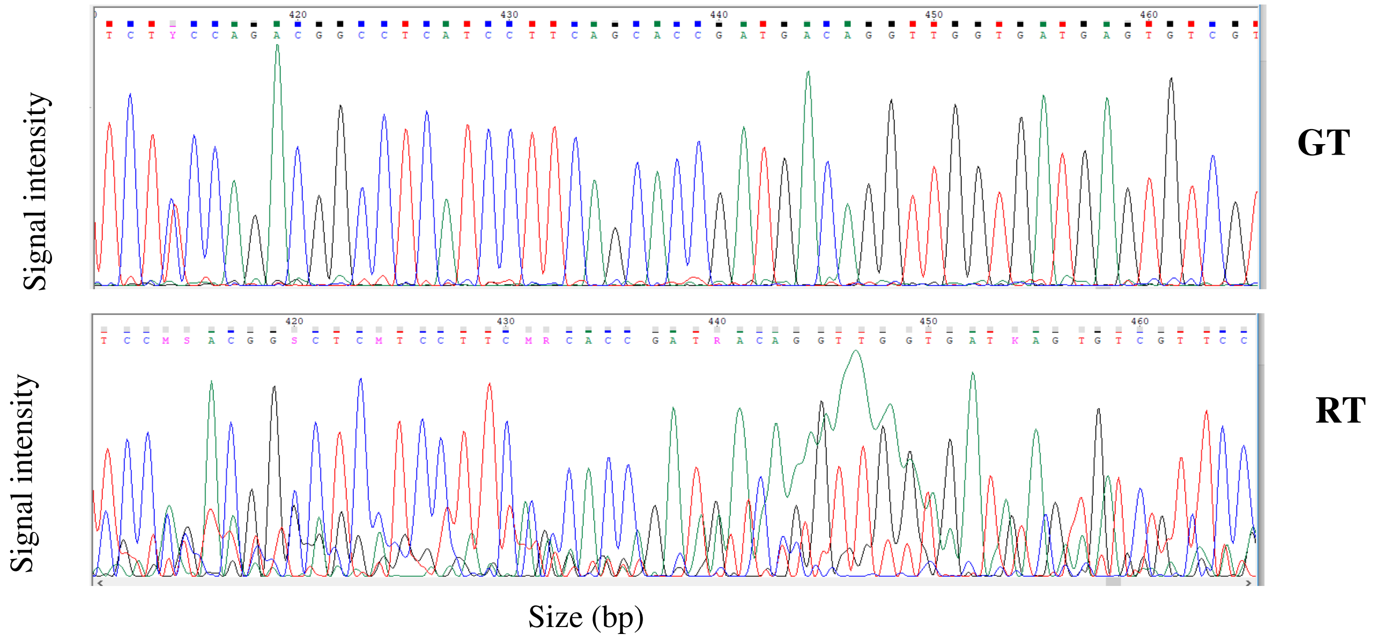


Figure C4: A portion of DNA sequence for exon 8 for grey and red-top samples. The figure shows the reverse complements for red and grey top samples. *Following DNA extraction, five targets of CYP2D6 were amplified using red and grey post-tox samples for case 22. The samples were then sent to the Central DNA Sequencing Facility at the University of Stellenbosch for sequencing.*

Exon 9: Forward orientation

Signal intensity

GT

Signal intensity

RT

Size (bp)

Reverse orientation

Signal intensity

GT

Signal intensity

RT

Size (bp)

Figure C5: A portion of DNA sequence for exon 9 for grey and red-top samples. The figure shows the forward reverse complements for red and grey top samples. *Following DNA extraction, five targets of CYP2D6 were amplified using red and grey post-tox samples for case 22. The samples were then sent to the Central DNA Sequencing Facility at the University of Stellenbosch for sequencing.*

APPENDIX D: Primary PCR for red-top tube samples (case 22)

M A B C D E

Figure D1: Primary PCR for red-top tube samples. *PCR for red-top samples using 5 µl of DNA template resulted in faint bands for exon 1.1: lane B. There was no visible band for exon 8: lane D. Exon 1.3 and 9 produced visible bands (lane C and E, respectively).*

APPENDIX E1: Ethics approval letter for pilot study

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.tsama@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.

HREC 110/2017

APPENDIX E2: Ethics approval letter for follow-up study

Form FHS007: Amendment – study staff

HREC office use only (FWA00001637; IRB00001938)			
☐ Approved			
This serves as notification that all changes to the study staff and documentation described below are approved.			
Chairperson of the HREC signature		Date	21/10/2017

Principal Investigator to complete the following:

1. Protocol information

Date (when submitting this form)	20 October 2017
HREC REF Number	HREC: 110/2017
Protocol title	Post-mortem molecular and toxicological investigation: Exploring toxicity and genetic variation in deceased individuals at Salt River Mortuary
Protocol number (if applicable)	
Principal Investigator	Laura Heathfield
Department / Office Internal Mail Address	Reception; Division of Forensic Medicine and Toxicology, Falmouth level building (entrance 3 level 1), FHS, UCT, Anzio Road, Observatory

1.1 Does this protocol receive US Federal funding?	☐ Yes	☐✓ No

2.1 Staff changes (tick ✓)

Are new personnel being added to this research?	☐✓ Yes	☐ No
Are current personnel being removed from this research?	☐ Yes	☐✓ No
Is the principal investigator for this research being changed? If yes, please attach revised conflict of interest and PI declaration statements. (Refer: sections 7 and 8.3 in the New Protocol Application Form - FHS013)	☐ Yes	☐✓ No
Do the consent and assent forms need modification to reflect these staff changes? If yes, please attach copies of the revised forms, with all changes highlighted or tracked and listed in the documents for approval.	☐ Yes	☐✓ No

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FACULTY OF HEALTH SCIENCES
Human Research Ethics Committee

2.2 Amended study staff details

Title, first name, surname	Department/Division	E-mail	Role of new staff member
Fungisai Musiyandaka	Pathology / Forensic Medicine and Toxicology	MSYFUN001@myuct.ac.za	Researcher

3. List of documentation for approval

Please list below all staff documentation such as CVs, declarations, GCP certificates and revised consent forms which need approval. This information must correspond to all 'yes' answers in 2.1 above. This form will be signed and returned to the PI as notification of approval. Please add extra pages if necessary.

CV attached.

4. Signature

My signature certifies that I will maintain the anonymity and/ or confidentiality of information collected in this research. If at any time I want to share or re-use the information for purposes other than those disclosed in the original approval, I will seek further approval from the HREC.

Signature of PI		Date	20/10/2017