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Evaluating the impact of environmental tobacco smoke on biological age markers: a canine model

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Submitted in fulfilment of the requirements for the
Degree of Doctor of Philosophy

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Abstract

This thesis aimed to examine the impact of environmental tobacco smoke on biological markers of ageing in pet dogs. In order to achieve this, community-based dogs and owners were recruited, approximately half of whom lived in smoking homes and half non-smoking homes. Owners were asked to attend 2 appointments, 12 months apart. Questionnaire assessments of dog environmental tobacco smoke exposure were compared to biomarkers in hair of the dogs (Chapter 3). This gave an objective measure of exposure and hair nicotine concentrations reliably reflected information provided by owners. A qPCR technique was optimised to measure telomere lengths (Chapter 4). This was utilised to measure telomere lengths in leukocytes, buccal cells, cremaster muscle, vas deferens and epididymis samples from the study dogs. Owners were offered free-of-charge neutering for their pet, and the spare tissues used for these analyses. A negative relationship in leukocyte telomere length with hair nicotine was observed, among other changes (Chapter 5). mRNA levels of further biomarkers of ageing were measured in testes, as well as leukocyte global DNA methylation percentage (Chapter 6). Again, several significant relationships were found between tobacco smoke exposure markers and the biomarkers of ageing, including a significant negative relationship with CDKN2A expression with increased tobacco smoke exposure. Plasma testosterone and hair cortisol concentrations were measured. In addition, factors which related to weight gain after neutering were examined (Chapter 7). Increased cotinine concentrations in fur were significantly related to increased percentage weight gain. Several avenues for future research were generated, and many areas warrant further investigation.

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*You were my light when in the dark, my guide when hope was lost.
Without your love and support, this thesis would not exist.
You changed my life the moment you looked at me and raised your front paws
to ask me to take you home.*

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I, Natalie Hutchinson, declare that this thesis embodies the results of my own special work, that it has been composed by myself and that it does not include work forming part of a thesis presented successfully for a degree in this or another University.

Date _____

Signature _____

Abbreviations

µl	Microlitre
17βHSD	17 β hydroxysteroid dehydrogenase
3βHSD	3 β hydroxysteroid dehydrogenase
5-mc	5-methylcytosine
AHR	Aryl hydrocarbon receptor
AIC	Akaike information criterion
ANOVA	Analysis of variance
bp	Base pair
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CI	Cephalic index
cm	Centimetre
DNA	Deoxyribonucleic acid
EEPS	Estimated effective population size
ELISA	Enzyme-linked immunosorbent assay
ETS	Environmental tobacco smoke
kb	Kilobase
kg	Kilogram
ln	Natural log
LTL	Leukocyte telomere length
M	Molar
mg	Milligram
mJ	Millijoule
ml	Millilitre
N	Number

ng	Nanogram
nM	Nanomolar
p450 SCC	Cytochrome p450 cholesterol side chain cleavage
PCR	Polymerase chain reaction
pg	Picogram
qPCR	Quantitative polymerase chain reaction
qRT PCR	Quantitative reverse transcription polymerase chain reaction
ROC	Receiver operating characteristic
RNA	Ribonucleic acid
RTL	Relative telomere length
SIMD	Scottish Index of Multiple Deprivation
sqrt	Square root
StAR	Steroidogenic acute regulatory protein
TRF	Telomere restriction fragment
TRF1	Telomeric repeat-binding factor 1
TRF2	Telomeric repeat-binding factor 2
V	Volt

Chapter 1: Introduction

1.1 Smoking as a Public Health Issue

According to the World Health Organisation Global Report on Trends in Prevalence of Tobacco Smoke 2015, tobacco use is the most common preventable cause of death worldwide, with an estimated 6 million people dying from it each year. This includes 600,000 people estimated to die as a result of second hand smoke effects. In Scotland in 2009, 24.2% of all deaths were from causes attributable to smoking (ScotPHO, 2016). Among UK women, smokers lose at least 10 years of their lifespan compared to non-smokers (Pirie *et al.*, 2013).

The rates of smoking in the UK have declined over the past century. In 1948, the rate of tobacco use (including pipe and rolled tobacco) was as high as 80% in men (Forey *et al.*, 2012). Studies such as those by Doll and Hill, 1950 and English, Willius and Berkson, 1940 established links between smoking and lung cancer and coronary heart disease, respectively, around this period and numerous further disease associations have been found since (examined in Section 1.2.2). Currently, 21% of people aged 16 and over smoke in Scotland and 6% of children aged 0-15 years are exposed to environmental tobacco smoke (ETS) in their own home (The Scottish Government, 2015c). The Scottish Government has set a target to reduce the rate of smoking to less than 5% in Scotland by 2034. They want young people to not start smoking, for people to be protected from second hand smoke and to help people to stop smoking (The Scottish Government, 2013). While 68% of Scottish adults stated they wished to quit smoking, the Scottish Smoking Cessation Services report stated that only 5% of service users were known to have successfully quit at 12 months (many were lost to follow up), so there is a challenge to find ways to engage people and enable them to maintain their quit status (Information Service Division, 2015).

1.2 Tobacco smoke

When a cigarette is smoked, “mainstream smoke” (first hand smoke) is formed and inhaled by the smoker. Between puffs, the cigarette still produces smoke which is released into the environment. This smoke is known as “sidestream smoke” (Woodward and Al-Delaimy, 1999). The mainstream smoke (MS) exhaled by the smoker plus the sidestream smoke (SS) inhaled by other individuals (including pets) is known as “second hand smoke”. However, individuals do not stop being exposed to tobacco smoke constituents when the cigarette is extinguished. The MS and SS remain in the environment, and chemicals are adsorbed onto surfaces from which they may be re-emitted over time. This source of exposure is referred to as “third hand smoke”. Therefore, the exposure of non-smokers and pets to tobacco smoke is a combination of second hand and third hand smoke, which together is known as “environmental tobacco smoke” (ETS) (Baker and Proctor, 1990).

Cigarette smoke is a combination of an aerosol of liquid droplets (the particulate phase), semi-volatile components and a mix of gases. Figure 1.1 shows the general composition of cigarette smoke by percentage, and Table 1-1 gives the general composition of the particulate and vapour phases, by percentage.

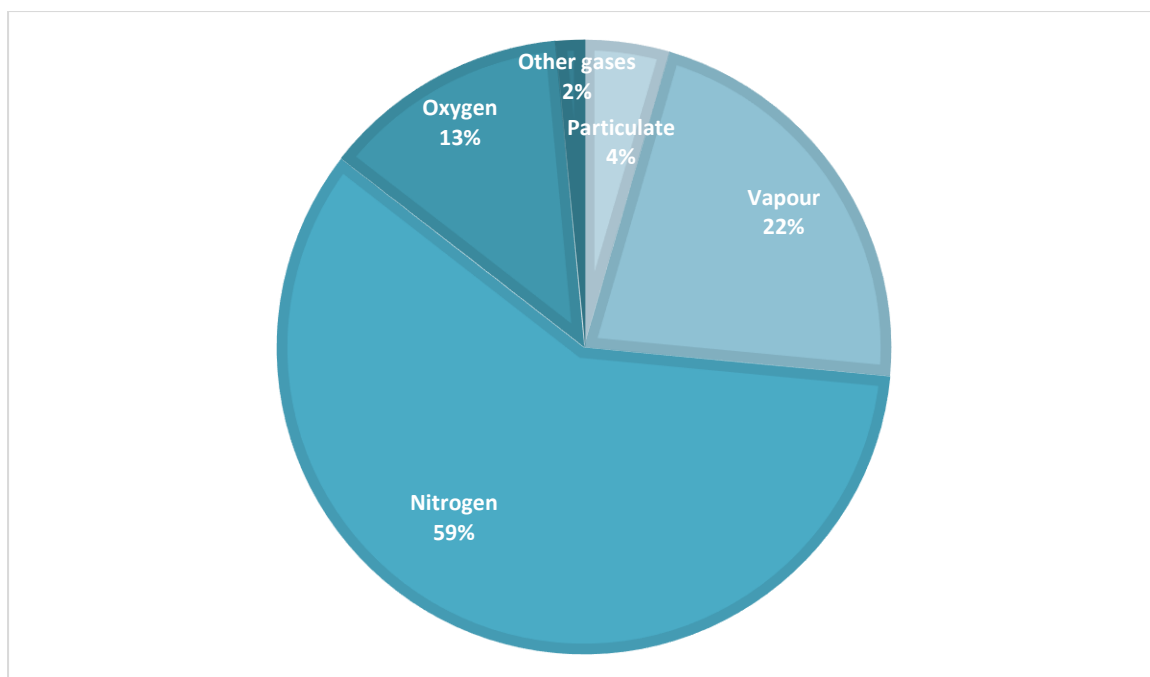


Figure 1.1 Cigarette smoke components by percentage
Adapted from Thielen, Klus and Mueller (2008)

Table 1-1 Components of the particulate and vapour phases of tobacco smoke
Adapted from Thielen, Klus and Mueller (2008)

Particulate phase		Vapour phase	
Component	Percentage	Component	Percentage
Water	16	Carbon dioxide	57
Alcohols and humectants	15.5	Carbon monoxide	18
Carboxyl acids	13	Water	18
Aldehydes and ketones	11	Hydrocarbons	3.5
Nicotine	6	Aldehydes and ketones	2
Alkanes	5	Nitriles	0.5
Terpenoid hydrocarbons	5	Miscellaneous	1
Pigments	4		
Phenols	3.5		
Esters	3.5		
Other alkaloids	3.5		
Miscellaneous and unidentified	14		

Over 5000 individual chemicals in cigarette smoke have been identified. Of these, the International Agency for Research on Cancer has classed 69 as animal carcinogens. These carcinogens include polycyclic aromatic hydrocarbons (PAHs) and tobacco specific nitrosamines (TSNAs). 12 of the PAHs in cigarette smoke are known carcinogens. The TSNAs NNN (N'-nitrosonornicotine) and NNK (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone) are classed as animal carcinogens and possible human carcinogens (WHO, 1986).

1.2.1 Biomarkers

Questionnaires are the most common method of assessing exposure to ETS (Woodward and Al-Delaimy, 1999). By using a biomarker, the absorbed dose of ETS in an individual can be measured. To be a successful biomarker, the measured variable should show a clear dose-response relationship, be able to demonstrate accurate exposure over a range of concentrations, and remain stable over time in a collected biological sample. In addition, the tissue sample for measurement should be easy to collect (Schick *et al.*, 2017). Many published studies involve measurement of biomarkers as an objective measure of a smoker's own exposure. The biomarker in this thesis needed to be present in tobacco smoke in sufficient quantity to be measured in non-smokers. Table 1-2 summarises examples of tobacco smoke biomarkers and example references using these.

Table 1-2 Tobacco smoke exposure biomarkers

Examples of tobacco smoke biomarkers in publications, with references split according to whether these were measured as a means of assessing the exposure of smokers or as an ETS biomarker (table structure adapted from Schick et al. (2017))

Tobacco smoke constituent	Example biomarkers	Example tissues used for measurement in publications	Example references: in smokers	Example references: ETS biomarker
Nicotine and metabolites	Nicotine Cotinine	Blood Urine Saliva Hair Nails Amniotic fluid	Kyerematen <i>et al.</i> , 1990; Eliopoulos, 1996; Jarvis <i>et al.</i> , 2008; Schütte-Borkovec <i>et al.</i> , 2009	Jarvis <i>et al.</i> , 1984; Al-Delaimy, Crane and Woodward, 2000; Al-Delaimy <i>et al.</i> , 2002; Kamer <i>et al.</i> , 2013; Stiby <i>et al.</i> , 2013
TSNAs	NNK NNAL	Urine Hair Nails	Stepanov <i>et al.</i> , 2007; Hu <i>et al.</i> , 2013	Anderson <i>et al.</i> , 2001; Hecht <i>et al.</i> , 2006; Pérez-Ortuño <i>et al.</i> , 2016
Volatile organic compounds	Benzene 1,3-Butadiene	Breath Urine	Carmella <i>et al.</i> , 2009; Kotapati <i>et al.</i> , 2011	Gordon <i>et al.</i> , 2002
PAHs	Pyrene 1-Hydroxypyrene	Urine	Astenf <i>et al.</i> , 2001; Feng and Liang Q, Kinser R,	Suwan-ampai <i>et al.</i> , 2009

			Jin Y, Scherer G, Urban M, Engl J, Riedel K, 2006; Scherer <i>et al.</i> , 2007	
Metals	Cadmium	Blood Urine	McElroy <i>et al.</i> , 2007	Willers, Gerhardsson and Lundh, 2005; Leung <i>et al.</i> , 2013
Carbon monoxide	Carbon monoxide Carboxyhaemo- globin	Breath Blood	Jarvis, Russell and Saloojee, 1980; Pojer, Whitfield and Richmond, 1984; Bailey, 2013	Jarvis, Russell and Feyerabend, 1983; Deveci <i>et al.</i> , 2004; Leitch <i>et al.</i> , 2005

1.2.1.1 Nicotine and metabolites

Only a small fraction of the many studies utilising nicotine and its metabolites are displayed in Table 1-2 (which only shows examples of studies in humans). Section 3.1.1 examines studies where these biomarkers have been measured in companion animals. Figure 1.2 displays the metabolism pathway of nicotine.

Nicotine is rapidly absorbed through the small airway in smokers and blood concentrations rise rapidly. Approximately 75% of nicotine is metabolised to cotinine by the enzyme P450 2A6 (Hukkanen, Jacob and Benowitz, 2005). Nicotine half-life in the blood of humans is 2 hours, whereas it was 53 minutes in Beagles exposed to cigarette smoke through a tracheostomy (Nightingale *et al.*, 1981). Nicotine is also readily absorbed in individuals exposed to ETS. SS is less acidic than MS, due to higher ammonia concentrations in SS, resulting in a higher proportion of nicotine being in its unionised form in SS (Woodward and Al-Delaimy, 1999).

A negative of using nicotine as a biomarker of ETS is that it is not carcinogenic, and therefore not necessarily measuring reflecting the risk of cancer in exposed individuals (Hecht, 2002). In addition, there are also various factors which affect the rate of nicotine metabolism, including age, renal function and medications affecting the activity of P450 2A6. In addition, both nicotine and cotinine metabolism are increased in females compared to males (Hukkanen, Jacob and Benowitz, 2005).

Due to the half-life of nicotine, measurements of concentrations of nicotine and derivatives in blood, urine and saliva represent short-term exposure. Hair and nails have been used as tissues for measurement of multiple substances, with compounds entering the hair via the follicle, apocrine glands and sebaceous glands, and being present on the shaft through external environmental exposure (Henderson, 1993). Hair has been described as a “tape-recorder” for dosage history, with a 1cm length of hair representing 1 months’ exposure in humans (Uematsu, 1993). The rate of growth of 1cm per month is similar to that of dogs (Gunaratnam and Wilkinson, 1983), with some potential variability with breed. Examples of utilisation of hair nicotine and metabolites as a biomarker for ETS exposure, and comparison with concentrations in other tissues are described in Chapter 3.

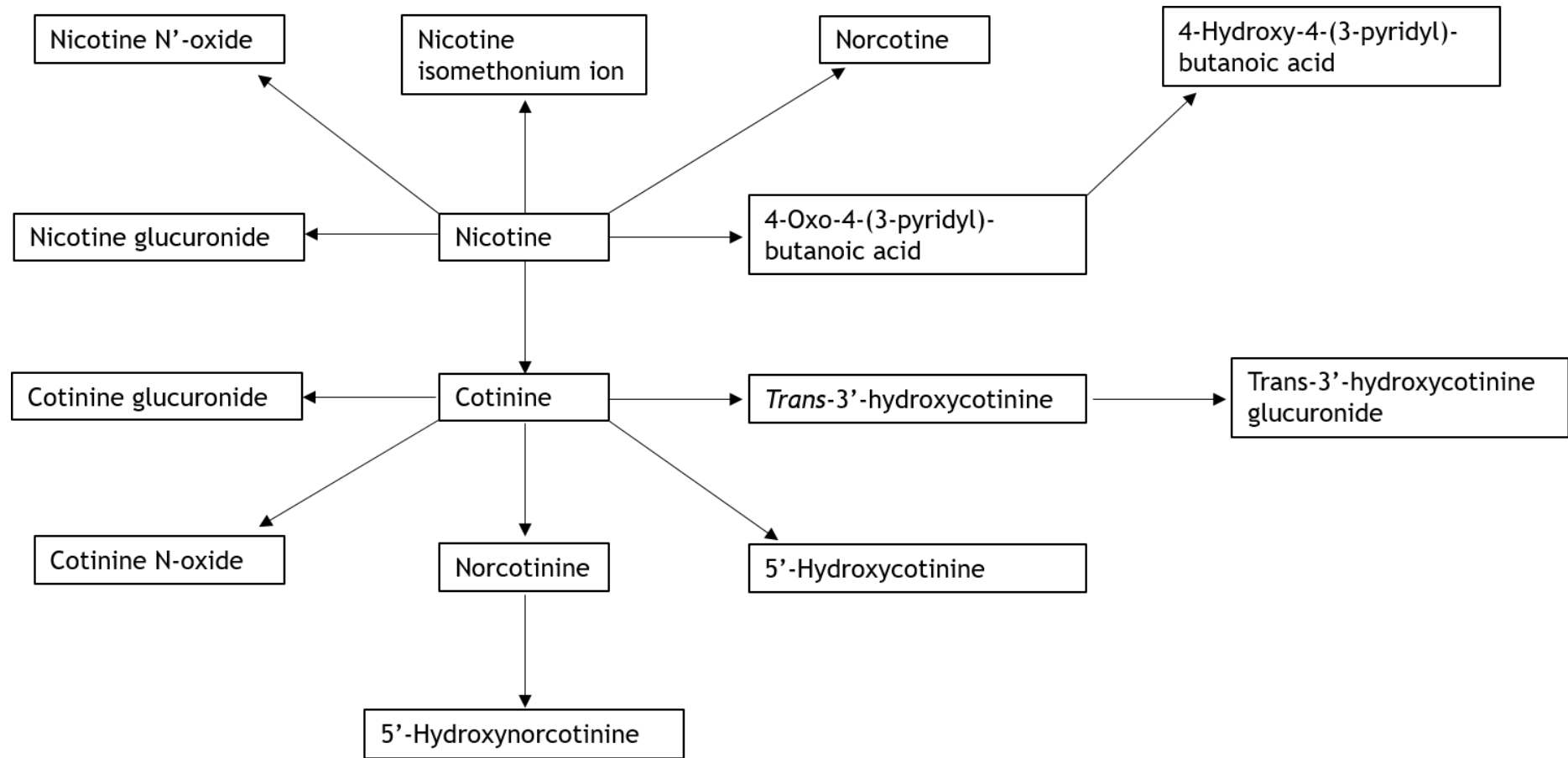


Figure 1.2 Nicotine metabolism pathways
Diagram adapted from Hukkanen, Jacob and Benowitz, (2005)

1.2.1.2 TSNAs

4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) is a metabolite of NNK, and is a carcinogen. The half-life of NNAL in humans is 10-18 days, so this marker maintains a steady level for biomarker analysis. However, the concentration of NNAL is very low, which may prove problematic for ETS exposure measurement. Hu *et al.* (2014) measured the concentration of NNK and NNAL in the urine of 101 smokers and 40 non-smokers. NNK and NNAL were not detectable in any of the samples from non-smokers and NNK was not detectable in the urine of the smokers. NNAL was detectable in 100 of the smokers' urine samples, and was significantly correlated with nicotine and cotinine concentrations. In order to measure long term exposure to NNN, NNK and NNAL, Pérez-Ortuño *et al.* (2016) measured concentrations in 48 hair samples, 24 from people exposed to ETS at home, 24 not exposed. NNAL was not quantifiable in any of the samples and NNN was only quantifiable in 7 ETS exposed and 2 non-exposed samples. NNK was quantifiable in 15 of the hair samples from ETS exposed people and 11 from non-exposed, and was highly correlated with nicotine and cotinine concentrations. In addition, nicotine, cotinine and NNK concentrations were all significantly higher in samples from ETS exposed than non-ETS exposed people. However, concentrations of NNK was very low. In ETS-exposed samples, median NNK was 1.3pg/mg, compared to 2040pg/mg nicotine and 49pg/mg cotinine. In non-ETS exposed samples, median NNK concentration was only 0.74pg/mg.

1.2.1.3 Volatile organic compounds

Volatile organic compounds (VOCs) are formed from incomplete combustion of organic materials, and physiologically from inflammation and lipid peroxidation (Gordon *et al.*, 2002; Statheropoulos, Spiliopoulou and Agapiou, 2005). Therefore, tobacco smoke is only one source of exposure. Several VOCs are harmful to health, and have been demonstrated to be increased in smokers compared to non-smokers. Kotapati *et al.* (2011) measured the concentration of a urinary metabolite of 1,3-Butadiene, a known carcinogen, in 27 smokers and 19 non-smokers. The concentration was 21.6ng/mg in smokers compared to 13.7ng/mg in

non-smokers. While the concentrations in the groups were significantly different, the data suggested that the non-smokers were being exposed from another source than tobacco smoke, for example car exhaust fumes.

1.2.1.4 Polycyclic aromatic hydrocarbons

PAHs are formed during incomplete combustion. They are found in tobacco smoke, air pollution, food, pesticides and industrial workplaces (Van Delft *et al.*, 2001; Lodovici *et al.*, 2004). Some of the higher molecular weight PAHs are known carcinogens, so measurement of these may provide useful information of the health risk of exposed individuals. However, concentrations in the body are low and the half-life of the metabolites is short, so concentration reflects short-term exposure. In addition, non-carcinogenic PAHs have been shown to be more strongly associated with reported tobacco smoke exposure than carcinogenic PAHs (Suwan-ampai *et al.*, 2009).

1.2.1.5 Metals

Metals in cigarette smoke include aluminium, cadmium, chromium, copper, lead and mercury (Bernhard, Rossmann and Wick, 2005). Lead exposure has been linked to illnesses such as cataracts and hypertension (Korrick *et al.*, 1999; Schaumberg *et al.*, 2004). Cadmium has been associated with renal disease and increased risk of various malignancies including lung cancer and breast cancer (Savolainen, 1995; McElroy *et al.*, 2006; Nawrot *et al.*, 2006). Leung *et al.* (2013) measured concentration of lead, cadmium and cotinine in urine samples from 893 children aged 2 to 7 in Hong Kong. 38% of children were exposed to ETS at home. Lead concentration was measurable in 3.9% of samples, whereas cadmium was measurable in 92.4%. Cotinine concentration was significantly higher in exposed compared to non-exposed children, and correlated with increasing number of cigarettes smoked at home. There was no significant difference in cadmium concentration between the exposure groups, and cadmium did not correlate with cotinine concentration. Whereas, in a study of 23 Swedish children of mean age

8.4, concentration of urinary cadmium was significantly correlated with urinary cotinine, and there was a non-significant positive correlation between urinary lead and cotinine (Willers, Gerhardsson and Lundh, 2005).

1.2.1.6 Carbon Monoxide

Carbon monoxide (CO) is formed as a result of biological processes and incomplete combustion of organic materials. CO competes with oxygen for haemoglobin binding and CO has a higher affinity for haemoglobin than oxygen. CO shifts the oxygen-haemoglobin dissociation curve to the left, impairing release of oxygen into tissues. Chronic exposure to CO from smoking has been linked to cardiovascular disease (Astrup and Kjeldsen, 1979).

Published studies examining CO as a biomarker for tobacco smoke exposure either involve measuring the concentration of CO in expired air or the concentration of carboxyhaemoglobin (COHb) in blood. COHb has a short half-life (2 to 3 hours in humans) and the vast majority of studies using this biomarker are as a means of quantifying exposure in the smoker rather than as a biomarker of ETS (e.g. Jarvis, Russell and Saloojee, 1980; Deveci *et al.*, 2004; Bailey, 2013). The measurement of CO in breath in human studies required the user to inhale deeply and exhale on command, so would not be practical for studies in this thesis.

1.2.2 Tobacco smoking and health

In the 15th century, Christopher Columbus brought the first tobacco leaves to Europe. Tobacco use was recommended by doctors for managing a range of ailments including toothache and worms (Musk and De Klerk, 2003). Associations between snuff, pipe smoking and cancer were suggested by individuals during the following centuries, but were disregarded by the wider community. Evidence began to mount for a link between morbidity and smoking in the first half of the 20th century. These reports were generally of incidental findings, such as that

patients with oral or respiratory cancers were often heavy smokers. For example, Abbe (1915) reported that in 100 private patients with cancer of the mouth, 89 were heavy smokers.

Case controls studies were needed to provide evidence beyond incidental findings that smoking was associated with increased risk of morbidity. The first case-control study regarding lung cancer mortality was credited to Müller in 1939. The findings of the study together with the rising incidence of lung cancer in the general population at the time, led Müller to conclude that tobacco smoking was the most important cause of the disease. Further case-control studies were undertaken to demonstrate the carcinogenic nature of smoking. Schrek *et al.* (1950), for example, found a higher incidence of smoking in a group of men with cancer of the lung or larynx compared to control patients with miscellaneous tumours.

Doll and Hill in 1950 presented a key paper in the British Medical Journal linking smoking to the increased number of deaths from lung cancer in the preceding 25 years, which had been recorded to increase from 612 to 9,287 per year. Following this article, Doll organised a prospective cohort study of smoking and mortality in UK doctors. Questionnaires were sent to doctors on the medical registry. As early as 2.5 years in to the study, a link was apparent between mortality due to lung cancer and smoking (Doll and Hill, 1954). Cause-specific mortality was followed up for 50 years, displaying a link between smoking and mortality due to vascular, neoplastic and respiratory diseases (Doll *et al.*, 2004).

Current estimates are that tobacco smoking is responsible for a third of cancer deaths in Western countries (Sasco, Secretan and Straif, 2004). The International Agency for Research on Cancer Working Groups have agreed that there is evidence for an increased risk of cancer associated with tobacco smoking in humans in the following tissue sites: lung, oral cavity, oro- and hypo-pharynx, oesophagus,

larynx, nasal cavity, paranasal sinuses, stomach, liver, pancreas, kidney, urinary tract, uterine cervix and myeloid leukaemia (WHO, 1986).

Alongside with the link between smoking and malignancy, in the early 20th century associations were being formed between coronary disease and smoking. Hoffman (1920) examined statistics of causes of death between 1900 and 1918, using data from the United States census office. An increase in the annual rate of death from heart disease was noted, on a background of a stable overall rate of mortality. English, Willius and Berkson in 1940 examined records of 1000 patients with coronary heart disease in relation to smoking, and found a link between the development of disease under the age of 50 with smoking.

Chronic obstructive pulmonary disease (COPD) is characterised by airflow limitation, which is progressive. There is an abnormal inflammatory response to noxious particles or gases. Smokers have more respiratory symptoms and lung function abnormalities than non-smokers, and an increased risk of mortality from COPD (Pauwels and Rabe, 2004). A prospective study of COPD in London working men was performed by Fletcher and Peto (1977). They found that in some smokers, an airflow limitation plus hypersecretory disorder was present, including infective processes. More recently, COPD has been cited as the fifth most common cause of death in high income countries, equating to 3.8% of deaths. Tobacco smoking is the most important cause of COPD, with smoking causing 73% of COPD-related mortality in high income countries.

With the rise of coronary heart disease and strokes in the 20th century, multiple cohort studies were established in attempt to discover the causes of the disease. The Framingham Heart Study is an American study which began in 1948. A cohort of 4,255 men and women aged 36 to 68 from this study were examined. In a 26 year follow up, 459 strokes occurred, with smoking being significantly related to the risk of stroke after age and hypertension were taken into account. The risk of

stroke increased with daily cigarette consumption (Wolf *et al.*, 1988). In a further study, 1,838 men from the Framingham Heart Study and another cohort study, the Albany Cardiovascular Health Center Study, were followed for 8 years. The men who smoked at least 20 cigarettes per day had a three times risk of myocardial infarction compared to non-smokers or former smokers (Doyle *et al.*, 1964).

Smoking has been associated with multiple other disorders of various body systems, for example, peptic ulcer (Kurata and Nogawa, 1997) psoriasis (Fortes *et al.*, 2005), vascular dementia (Anstey *et al.*, 2007), cataracts (Ye *et al.*, 2012), periodontitis (Bergström, 2006), and male and female infertility (Vine, 1996; Augood, Duckitt and Templeton, 1998). Birth defects have also been associated with maternal smoking in pregnancy. Hackshaw, Rodeck and Boniface (2011) examined birth data from 1959 to 2010, including 173,687 cases of birth defects and 11,674,332 unaffected controls. Significant positive associations were found between maternal smoking and orofacial clefts, clubfoot, cardiovascular/heart defects and gastrointestinal defects in the infant.

1.3 ETS exposure and dog health

Studies regarding examples of increased risk of poor health in dogs exposed to ETS are few in number. While it would be assumed that dogs are at risk from ill health from ETS the same way that humans are, there are few studies documenting associations between exposure and disease in dogs.

Hawkins *et al.* 2010 used questionnaire and historical data from clinical records to try to ascertain risk factors associated with chronic cough in dogs. The dogs in question had presented to a veterinary hospital with at least a 2-month history of cough. The authors did not find a relationship between ETS exposure and cough. In addition, none of the 6 cases that were diagnosed as having cancer were from smoking homes. Whereas, when 43 dogs with chronic cough were studied in a

Japanese veterinary centre, indices of airway limitation were significantly higher in dogs exposed to ETS (Yamaya, Sugiya and Watari, 2014). When 30 Yorkshire terriers, half of which lived in a smoking home, underwent bronchiolar lavage, macrophage and lymphocyte numbers were observed to be significantly higher in the fluid obtained from dogs in smoking homes, and anthracosis was present in macrophage cytoplasm in 8 of the 15 ETS exposed dogs (Roza and Viegas, 2007).

Reif *et al.* (1998) conducted a case-control study in nasal cancer of dogs, examining whether there was a difference in risk of developing nasal cancer in smoking homes compared to a control group, which had other types of cancer. They found an increased risk of nasal cancer with ETS exposure, but only in long nosed breeds with the highest level of exposure, as determined from questionnaire data. A case control study on lung cancer in dogs relating to ETS exposure, again using other cancer cases as the control group, concluded that the effect of ETS on risk of lung cancer was restricted to short and medium length nosed dogs. However, the quoted 95% confidence interval quoted for the odds ratio result on which they based their claim crosses 1.00, so the conclusions they reach read as somewhat confusing (Reif *et al.*, 1992). Glickman *et al.* (1989) interviewed owners of 59 dogs with transitional cell carcinoma of the bladder and owners of 71 dogs with chronic diseases. The odds ratio of having bladder cancer if the dog lived in a smoking home was not significant.

In addition to the effects of ETS, dogs may consume whole cigarettes, cigarette butts discarded on the floor or the contents of ashtrays in the home. Novotny *et al.* (2011) collected data from the Pet Poison Help Line, Bloomington MN and ASPCA Animal Poison Control Center, Urbana IL and found over the period of 2005-2010 there were 801 calls related to dogs consuming cigarettes or butts. 55% of the dogs experienced vomiting and 25% ataxia. There was one reported case of death, however it was unclear if the dog was euthanized for reasons related to cigarette consumption.

1.4 Dogs as sentinels of exposure

Pet dogs share their owner's home environment, without engaging in behaviours such as alcohol consumption which may confound epidemiological studies. They often spend nearly all their lives in one home, which may be a better example of "real world" exposure than that which is regulated within a laboratory. Dogs have been used as sentinels for exposure to many potentially harmful agents in previous studies, including mercury (Dunlap *et al.*, 2007), and herbicides (Glickman *et al.*, 2004). In addition, dogs spend a lot of their time at home, so potentially would be exposed to third-hand smoke in a similar nature to young children. Like small children, they often explore their environment with their mouths, potentially ingesting third-hand smoke and increasing their exposure. In addition, being quadrupeds, they spend a lot of time close to the floor. Matt *et al.* (2004) conducted a study to examine ETS contamination in household air, dust and on surfaces in homes of non-smokers, smokers and smokers who try to protect their families by only smoking outside or away from others (indirect exposure group). The study found that in the living room of the houses, the indirect exposure group had significantly higher air nicotine, surface nicotine and nicotine dust levels than the non-exposure group and the direct exposure group had significantly higher levels than the indirect. This showed that while people in the indirect group reduced the exposure of their families to nicotine compared to those who smoke inside, they did not protect them completely. In fact, average nicotine levels in the homes of the indirect group were 5-7 times higher than the non-exposure group. The study also examined the levels of nicotine in the mothers and children in the households. No nicotine was detected on the fingers of mothers in the non-exposure group, but nicotine was detected on 100% and 92% of the fingers of mothers in the indirect and direct groups, respectively. A higher concentration of nicotine was found on the fingers of mothers than the household surfaces. Exposure to ETS in the children was confirmed using concentrations of nicotine and cotinine in urine and hair samples. This confirmed that there were significantly higher levels of exposure in the indirect group compared to no exposure, and significantly higher levels in the direct compared to indirect group.

Dogs are also a useful sentinel for ETS exposure due to there being an estimated 8.5 million dogs in UK households (24% of households), with 46% of households in the UK owning any type of pet (Pet Food Manufacturers' Association, 2015). Many elderly people live alone, save the company of a pet and may wish to protect their pet from smoke (Hovell and Irvin, 2007). Such people, and younger single childless pet owners, are not targeted in campaigns against child exposure to ETS. A web-based survey of 3293 adult pet owners in Michigan with questions about smoking behaviour of owners and their cohabitants was conducted. 21% of people in the survey were smokers, which as previously mentioned is the current rate of smoking in Scotland. In addition, 76% of respondents were dog owners. 28.4% of smoking pet owners stated that information on the dangers of second hand smoke to their pets would motivate them to quit smoking, and 14.2% said they would potentially only smoke outside. Provided with similar information, 16.4% of non-smoking respondents said they would ask their co-habitants to quit smoking, and 24.2% would ask people to go outside to smoke (Milberger, Davis and Holm, 2009).

1.5 What is a biomarker of age?

Age usually refers to the chronological length of time since birth, with older age leading to a progressive decline in physiological function and higher risks of age-related morbidity and mortality. Whereas, “biological” age refers to the physiological function of an individual relative to normal physiological progression throughout lifespan (Flatt, 2012; Jarman *et al.*, 2015).

Baker III and Sprott (1988) proposed a case for identifying biomarkers which would better represent physiological function than chronological age. They laid out a set of criteria which the biomarker should meet:

- The rate of change of the biomarker should reflect a parameter which can be predicted at a later stage

- The biomarker should reflect a biological process of ageing rather than a predisposition to disease
- The biomarker should be reproducible cross-species
- The biomarker should change independently with chronological age
- The biomarker should be non-lethal to measure
- The biomarker should be measurable over a short time relative to the lifespan of the animal

Molecular age biomarkers (MABs) were evaluated in this thesis. MABs measure aspects of DNA and RNA abundance or sequence over the animal's lifespan (Jarman *et al.*, 2015).

1.6 Telomeres

In the late 1930s, work performed by Hermann Muller on fruit flies and studies by Barbara McClintock on maize led to the discovery of natural ends at chromosomes which prevented the joining of the end of chromosomes to each other. Muller named these ends “telomeres” (McClintock, 1931; Muller, 1938). Yet it was some time after this that the true structure and function of telomeres was discovered. In the 1970s, the sequence of telomeres in *Tetrahymena* yeast was described, a series of TTGGGG repeats, and subsequent work led to the finding of telomerase, an enzyme which could synthesise telomeric repeats *de novo* in this yeast in 1984 by Elizabeth Blackburn (Blackburn and Szostak, 1984). It was already known that DNA polymerase could only extend preformed DNA and so could not extend the very end of linear DNA (“the end replication problem”) (Watson, 1972). While the majority of DNA is double-stranded, the distal end contains a 3' overhang in the telomere region. Blackburn found that telomerase which could extend this overhang. It is now known that the overhang or “G-tail” is folded back on itself to form a “t-loop” to prevent enzymatic degradation and end-fusion events (Griffith *et al.*, 1999).

By the late 1980s, the human telomere sequence was established to be repeats of TTAGGG (Moyzis *et al.*, 1988), only slightly different to that of yeast, and telomerase was found in human cells, opening up a whole avenue of new research into cellular senescence and cancer. Harley, Futcher and Greider (1990) examined human cells in culture and found that telomere shortening occurred with each cell division due to the end replication problem with a lack of telomerase expression. Senescence by activation of the p53 checkpoint system occurred when a critically short telomere length was reached, which defined a finite number of times a cell can divide, the so called “Hayflick Limit” (Hayflick and Moorhead, 1961). If telomerase was expressed, telomeres did not shorten and the cells did not show senescence. Although cells after passing the Hayflick limit can still live for a long time, they cannot be stimulated to proliferate again except by transformation. However, in the presence of somatic mutations or viral oncogenes, the cellular senescence triggered by telomere shortening may be bypassed, so the cell can continue to divide and the telomeres continue to shorten. Continued telomere erosion can lead to the activation of telomerase, maintaining the remaining telomere and the cell can continue to proliferate. An alternative method of evasion of telomere induced senescence in tumours and cell lines has been observed, the Alternative Lengthening of Telomeres (ALT) (Shay and Wright, 1996; Bryan and Reddel, 1997).

Telomeres have a protective function. The loss of telomeres has been demonstrated to be invariably associated with Robertsonian chromosome rearrangements in mice. Robertsonian rearrangements involve centromere fusion of two telocentric or acrocentric chromosomes to form a single metacentric chromosome. In telocentric chromosomes, the centromere is located at the terminal end of the chromosome, and these type of chromosomes are present in mice (Hemann *et al.*, 2001). All autosomal chromosomes in the dog are acrocentric, where the P-arm (short arm) of the chromosome is very short, but longer than in telocentric chromosomes (Oshimura, Sasaki and Makino, 1973). In the telocentric chromosomes of mice, most Robertsonian fusion events involve two

P-arms, emphasising the importance of telomere function at P-arms to prevent these fusion events from occurring (Slijepcevic, 1998).

Telomeres must avoid having the 3' overhang being recognised by the cell as a broken DNA end and therefore stimulating DNA damage pathways. A group of telomere associated proteins collectively known as shelterin provides this function. Shelterin proteins form t-loops at the end of telomeres which result in the end in the end of the telomere being tucked in and the 3' overhang invading the double stranded region forming a D-loop (Figure 1.3) (de Lange *et al.*, 1990; O'Sullivan and Karlseder, 2010).

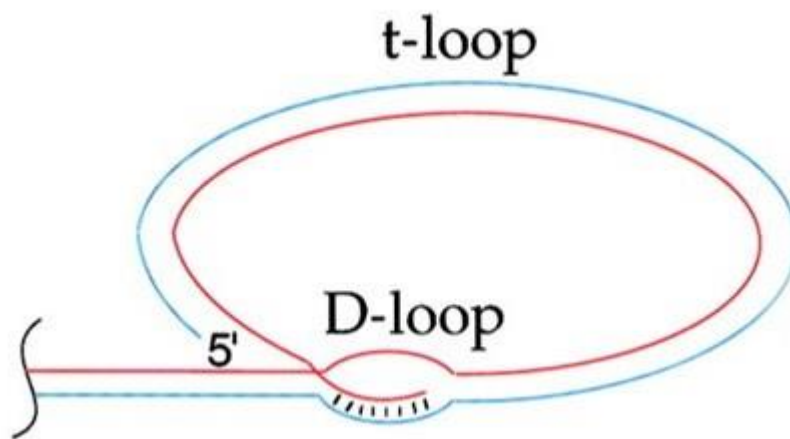


Figure 1.3 Telomeres have a t-loop and D-loop at the end
Copied from Greider (1999) with permission

Shelterin includes the following proteins: telomeric repeat binding factors 1 and 2 (TRF1 and TRF2), TRF1 interacting protein 1 (TIN2), repressor/activator protein 1 (RAP1), protection of telomeres 1 (POT1) and TPP1 (also known as TINT1/PIP1/PTOP1). TRF1 and TRF2 bind the double stranded repeat, POT1 attaches to the 3' overhang, and these proteins are then bridged by TPP1, TIN2 and RAP1 (De Lange, 2005; O'Sullivan and Karlseder, 2010). *In vitro* study of TRF2 demonstrated that a dominant negative allele of TRF2 induced end-to-end chromosome fusions (Van Steensel, Smogorzewska and De Lange, 1998). In

addition, *in vitro* targeting TRF1 and TRF2 individually lead to telomere shortening, demonstrating that these proteins act together (Ancelin *et al.*, 2002).

Following the observations by Harley, Futcher and Greider (1990) that telomeres shortened in human fibroblasts with increasing population doublings, telomere length was measured in human blood samples to test if there was a negative relationship with age. Hastie and Dempster (1990) measured telomere length in 47 human blood samples. There was a significant negative relationship with age observed, equivalent to 33bp per year. In a further study using blood samples from 140 humans aged 0 to 107 years, telomere length declined by 41bp per year (Vaziri *et al.*, 1993). A negative relationship between telomere length in blood samples and age has been found in many human studies, and the shortening of telomeres with each cell division has been said to act as a “biological clock” (Von Zglinicki, 1998).

As with studies in human fibroblasts, when canine dermal fibroblasts were cultured *in vitro*, telomere length declined with population doublings, from 20.5kb at passage 1 to 17.5kb at passage 16 (Nasir *et al.*, 2001). Also, as with humans, the relationship between telomere length in leukocytes and age has been examined in dogs. For example, Fick *et al.* (2012) measured leukocyte telomere length (LTL) in 175 dogs of various breeds and found a significant decline with age, at a rate of 360bp per year. McKeivitt *et al.* (2002) measured LTL in 47 dogs aged 6 months to 13 years. There was a negative non-significant trend with age.

1.7 Telomeres and oxidative stress

In telomerase-negative cells, telomeres shorten with each cell division. However, the rate of telomere shortening is not constant. The rate is influenced by the degree of oxidative stress and the level of antioxidant defence. Some of the oxidative damage inflicted upon telomeres cannot be repaired and the damaged

DNA is lost in the next replication (von Zglinicki, 2002). As telomeres contain high levels of guanine residues, they are particularly sensitive to oxidative stress damage. Guanine residues have the lowest oxidation potential of the 4 bases in DNA, so are most likely to be damaged by reactive oxygen species (ROS) and they are even more vulnerable to oxidative reactions when in triplicate as occurs in telomeres. ROS can modify guanine bases into 8-oxo-7, 8-dihydroguanine (8-oxodG), which can induce single strand breaks. Oxidative damage cumulates over the life span of a cell. Senescent cells have 30% more oxidatively changed guanine bases and higher levels of 8-oxodG. A single 8-oxo-dG lesion can reduce the percentage of bound TRF1 and TRF2 proteins by at least 50%, and the effect increases with more lesions (Opresko *et al.*, 2005).

By looking at the rate of loss of telomere bases, inferences can be made as to the level of oxidative stress upon the whole genome (von Zglinicki, 2002), and thus the environmental influences upon the individual. Research has demonstrated that cigarette smoking induces oxidative stress. In vitro experiments by Carnevali *et al.* (2003) showed that cigarette smoke extract induced oxidative stress and apoptosis in human lung fibroblasts. This relationship was dose-dependent. Keaney Jr *et al.* (2003) demonstrated that in humans cigarette smoking was significantly associated with increased urinary 8-epi-PGF_{2α}, a marker of systemic oxidative stress. Demissie *et al.* (2006) found that this marker of oxidative stress in urine was inversely related to telomere length in humans. In addition, passive smoking has been shown to cause oxidative stress in children (Demissie *et al.*, 2006). Plasma peroxide levels were found to be significantly higher and measures of antioxidant levels significantly lower in children exposed to passive smoking compared to those who were not (Kosecik *et al.*, 2005).

1.8 Factors in individuals related to telomere length

1.8.1 Lifespan and telomere length

Dogs are a unique species in that there is such a wide variety of life expectancy and size within the species. Fick *et al.* (2012) found that in 175 dogs, LTL was strongly positively predictive of breed life span. However, across species, telomere length was negatively correlated with life span in an analysis of over 60 mammalian species (Gomes *et al.*, 2011). This was not simply due to a relationship with size, as there was no independent association between body size and telomere length observed. Gomes *et al* also examined differences in patterns of telomerase activity across species. In smaller mammals, such as the house mouse and Norway rat, telomerase was active across a range of tissues. Whereas, in larger mammals, such as dogs and humans, telomerase was repressed in tissues, and active in only a small number of cell types, such as germ cells. Telomerase is highly active in testes in humans, and unlike many tissues, telomeres have been found to increase in length in sperm (Eisenberg, Hayes and Kuzawa, 2012). Examples of species telomere length and telomerase pattern are shown in Table 1-3.

Larger mammals tend to have repressed telomerase, short telomeres and use “replicative ageing”, where short telomeres result in growth arrest *in vitro*. Species not using replicative ageing have a higher replicative capacity, but are at increased risk of tumorigenesis. The response to damaged telomeres also varies between small and large species. In humans, critically short telomeres induce senescence through the p53 or p16/retinoblastoma pathway. Suppression of both of these pathways is required to suppress senescence (Ben-Porath and Weinberg, 2005). In *in vitro* experiments of mouse cells, telomere damage also resulted in senescence but this senescence could be prevented by the loss of p53 function, indicating that the p16/retinoblastoma pathway is not linked to telomere dysfunction in mice (Smogorzewska and de Lange, 2002).

A relationship between telomere bases lost per year of age and lifespan has also been observed, with greater yearly losses being associated with shorter lifespans (Haussmann *et al.*, 2003). In addition, with the same species of bird, the Alpine swift, it was demonstrated by examining telomere length over a 6 year period that those with long telomeres and slow rate of yearly loss had higher rates of survival and longer lifespans (Bize *et al.*, 2009).

Table 1-3 Examples of telomere restriction fragment lengths and telomerase in various species
The species, published telomere length and whether telomerase is restricted in somatic tissues is given. * indicates the telomere lengths in the study went above the limits of the authors' assay, so the true upper length is not known.

Species	Telomere length	Telomerase restricted	Reference(s)
House mouse (DBA/2, CBA/Ca, C57BL/6J laboratory strains)	Up to 150kb*	No	Kipling and Cooke, 1990; Broccoli <i>et al.</i> , 1996
Chicken	8 to 20kb	No	Venkatesan and Price, 1998
European White rabbit (inbred laboratory strain)	10 to over 80kb*	No	Forsyth <i>et al.</i> , 2005
Domestic cat	4.7 to 26.3kb	Yes	McKevitt <i>et al.</i> , 2003
Domestic dog	10.9 to 23.5kb	Yes	Nasir <i>et al.</i> , 2001; Yazawa <i>et al.</i> , 2001; McKevitt <i>et al.</i> , 2002; Cadile <i>et al.</i> , 2007; Benetos <i>et al.</i> , 2011
Cynomolgus monkey	15.4 to 17.5kb	Yes	Gardner <i>et al.</i> , 2007
Human	4.5 to 18.9kb	Yes	Kim <i>et al.</i> , 1994; Iwama <i>et al.</i> , 1998; Melk <i>et al.</i> , 2000; Okuda <i>et al.</i> , 2002; Kimura <i>et al.</i> , 2008

1.8.2 Breed, weight and neutering as determinants of longevity

Several studies have found a link between increased average dog breed weight and decreased life expectancy (Li *et al.*, 1996; Patronek, Waters and Glickman, 1997; Galis *et al.*, 2007; Greer, Canterbury and Murphy, 2007; Adams *et al.*, 2010; Dreschel, 2010; O'Neill *et al.*, 2013). For example, when data was collected from over 700 dogs of 77 breeds, there was a significant negative correlation between longevity and both height and weight, with weight being a strong predictor of lifespan (Greer *et al.*, 2006). Interestingly, (Galis *et al.*, 2007) observed that while there was a negative correlation between size and longevity overall, within breeds there was a positive correlation between weight and longevity. In addition, a further study found that within weight categories, cross breed dogs had a longer median lifespan than purebred dogs (Patronek, Waters and Glickman, 1997). Cross breeds lived longer on average than pure breeds overall in two surveys of British mortality data (Michell, 1999; O'Neill *et al.*, 2013). Dreschel (2010) replicated the weight and lifespan negative correlation and also found that neutered dogs lived significantly longer than entire dogs. Neutering was also found to give increased lifespan in a study using over 80,000 mortality records of animals presenting to North American veterinary teaching hospitals. This was the case for both sexes, with an increase of life expectancy of 13.8% in males and 26.3% in females (Hoffman, Creevy and Promislow, 2013).

1.8.3 Risk of mortality and telomere length

There is mixed evidence in humans as to whether an individual's telomere length is a predictor of likelihood of mortality. For example, when LTL was measured in 2744 men of mean age 75.5 and 20% died in the 6 year follow up period, there was no association between initial LTL and subsequent mortality (Svensson *et al.*, 2014). In addition, Bendix *et al.* (2014) found no association between LTL and mortality in the 10 year follow up in 1356 individuals aged 30 to 70. Whereas, when Fitzpatrick *et al.* (2011) measured LTL in 1136 participants of mean age 73.9, those with the shortest quartile of LTL were 60% more likely to die during the 6 year follow up than those with the longest quartile. In a further study, in 143

individuals aged over 60, shorter telomeres were significantly related to mortality in the following 15 years. This was found to be in part attributed to a 3 times higher mortality rate from heart disease and an 8.5 times higher mortality rate from infectious disease in those with the shortest quartile of LTL (Cawthon *et al.*, 2003).

Aside from predicting human mortality, telomere length was seen to be significantly associated with mortality in a closed wild population of Seychelles Warblers. This was in a well sampled population, where age was known for the birds. When 62 of 200 individuals died in the same year as last having a blood sample taken, telomere length, but not age, was a significant predictor of mortality, with those which subsequently died having shorter telomere length (Barrett *et al.*, 2013).

1.8.4 Morbidity and telomere length

Telomere length has been shown to be significantly shorter in humans with various diseases across multiple body systems compared to healthy counterparts. For example, rheumatoid arthritis, type 2 diabetes mellitus, liver cirrhosis, chronic heart failure, chronic obstructive pulmonary disease and multiple sclerosis patients were all shown to have shorter telomere lengths than healthy controls (Wiemann *et al.*, 2002; Steer *et al.*, 2007; van der Harst *et al.*, 2007; Albrecht *et al.*, 2014; Liu *et al.*, 2014; Guan *et al.*, 2015).

Cadile *et al.* (2007) measured the telomere length of 57 solid-tissue tumour samples, 40 normal tissue samples from 12 healthy dogs and 4 blood samples from blood donor dogs. The mean telomere length was 19.0kb in both the tumour samples and healthy tissue samples, however there was a larger variability in telomere length in the tumour samples. Yazawa *et al.* (2001) compared telomere length in 27 mammary gland tumour samples to 12 normal mammary gland samples and found there was no significant difference between the two groups. However,

in normal tissue, age was significantly negatively related to telomere length, whereas there was no significant association between age and telomere length in the tumour samples.

Aside from telomere length in tumours, no studies regarding telomere length and chronic disease in dogs could be found. However, an association between telomere length and chronic kidney disease (CKD) in cats has been documented (Quimby *et al.*, 2013). Telomere length was measured in samples of proximal and distal tubular epithelial cells from 12 cats with CKD, 12 young healthy cats of mean age 2.7, and 6 old healthy cats, with a mean age of 11.5. Reduced telomere length was observed in CKD cats compared to both the young and old healthy cats.

No studies could be found regarding telomere length and canine obesity. Although, with 20.4% of dogs being obese and 38.9% being overweight in 696 dogs attending five veterinary practices in the UK (Courcier *et al.*, 2010), it is worth considering adiposity as a potential factor for telomere length variation. Examples of the relationship between obesity and telomere length are described in Chapter 7.

1.8.5 Heritability of telomere length

Studies have been undertaken in humans examining to what extent telomere length is inherited and how much environmental factors influence, using twin studies and parent/offspring studies. Paternal age has been shown to positively relate to offspring telomere length (e.g. Eisenberg, Hayes and Kuzawa, 2012; Broer *et al.*, 2013). However, in terms of heritability of telomere length there is mixed evidence as to whether maternal or paternal telomere length has a stronger influence. Nawrot *et al.* (2004) found a strong correlation between father's and daughter's telomere length but not father's and son's, but a strong correlation between mother's telomere length and both son's and daughter's telomere length. This lead them to suggest an X-linked inheritance of telomere length. Whereas,

Njajou *et al.* (2007) observed a stronger relationship between paternal and offspring telomere length, than maternal and offspring telomere length. They also observed a positive relationship between daughter's telomere length and paternal lifespan, but not maternal lifespan.

In a study of 123 human monozygotic and dizygotic twins, results indicated that 78% heritability for LTL (Slagboom, Droog and Boomsma, 1994). Hjelmberg *et al.* (2015) measured LTL in 355 monozygotic and 297 dizygotic same-sex twins. They found a lower heritability estimate than Slagboom *et al.* of 64%, and 22% due to shared environment effects. Heritability of telomere attrition rate was estimated to be 28%, and 72% of attrition was estimated to be linked to individual environment effects. This may indicate that a large proportion of telomere length would be determined in the dogs in this thesis by genetic factors such as breed, but environmental factors like ETS exposure may have a larger determining role in telomere length attrition.

Aside from parental inheritance, dog breeds are genetically divergent and telomere length may vary between breeds. When LTL was measured in 22 Labrador retrievers, 17 miniature schnauzers and 8 beagles, a significant breed effect was observed (McKevitt *et al.*, 2002).

1.8.6 Sex and telomere length

A sex effect on telomere length has been found in many studies. Female human newborn LTL was significantly longer than male newborn LTL in 490 infants (Factor-Litvak *et al.*, 2016), and non-significantly longer in females in a further study on 168 infants (Okuda *et al.*, 2002). Female human adults are commonly found to have longer LTL than their male counterparts (e.g. Nordfjäll *et al.*, 2008; Ahola *et al.*, 2012; Steenstrup *et al.*, 2013; Chen *et al.*, 2014; Berglund *et al.*, 2016).

Two studies have described the relationship between sex and telomere length in dogs. Fick *et al.* (2012) found a significant negative relationship between LTL and age. However, when stratified by sex, the relationship was no longer significant in females, but was still significant in males. In contrast, in a recent study measuring LTL in 89 Chihuahuas, when the significant negative relationship with age was stratified by sex, the relationship only remained significant in females (Buddhachat *et al.*, 2017).

1.8.7 Cephalic Index

The cephalic index (CI) is a ratio of skull width to skull length. Brachycephalic dogs, i.e. those with a short muzzle, have a high cephalic index. For example, in breed data gathered at dog shows in Australia, pugs had a mean CI of 98.54, whereas greyhounds, a long nosed or dolichocephalic breed, had an average CI of 46.34 (McGreevy *et al.*, 2013).

No data could be found on telomere length and cephalic index. However, in a survey of purebred dogs in the UK, dogs with very high cephalic indexes appeared to have a lower median age of death, when compared to other dogs in their own weight category (five weight groups were used: toy, small, medium, large, giant). Pugs and French bulldogs, who have a high CI, were in the “small” weight group and were found to have a median age of death of 11 and 9 years, respectively. Other dogs in this weight category without a high cephalic index and corresponding median age of death included: dachshund, 12.67 years, Border terrier, 14 years, and Lhasa Apso- 14.33 years. In the medium weight group, the English bulldog, another high CI breed, had a median age of death of only 6.29 years of the 180 deaths surveyed, compared to 12.25 years in 106 Border collies (Adams *et al.*, 2010).

Brachycephalic dogs have been shown to be prone to upper respiratory disorders, specifically brachycephalic airway obstructive syndrome and gastrointestinal disorders, namely reflux oesophagitis and hiatus hernia (Koch *et al.*, 2003; Lecoindre and Richard, 2004). Chronic disease states have been linked to shortened telomere length. Dolichocephalic dogs have also been demonstrated to be prone to their own issues. As described in Section 1.3, dolichocephalic dogs were found to be at higher risk of nasal cancer when exposed to very high levels of environmental tobacco smoke, whereas short and mid length muzzle dogs were not (Reif, Bruns and Lower, 1998). It could be speculated that this may equate to these dogs being more sensitive to telomere erosion due to airborne environmental factors.

1.8.8 Inbreeding

In pure breed dogs, there is a limited sized population and therefore an inevitable degree of inbreeding. Selecting within breeds on the basis of certain desirable traits leads to further inbreeding, as two individuals are selected for this purpose are more likely to be related than two dogs selected at random (Lewis, Abhayaratne and Blott, 2015). Leroy *et al.* (2015) used French Kennel Club data to investigate the effect of inbreeding on 7 dog breeds. They found a negative correlation between degree of inbreeding and percentage who survived to 2 years of age.

In mice, when several in-bred and out-bred lines were compared, in-bred mice had significantly longer LTL than out-bred mice. If the mice were crossed, the offspring had LTL intermediate to that of the parents (Manning *et al.*, 2002). No correlation between LTL and inbreeding was found in a group of human subjects by Mansour *et al.* (2011), when DNA polymorphisms and family history were used to determine inbreeding.

The effective population size was a concept in genetics first discussed by Wright (1931). The population number is rarely directly proportional to the amount of genetic drift. The amount of genetic drift in a population is proportional to the effective population size. The population loses genetic diversity when the effective population size is less than 100 and the measure can be used as a marker of inbreeding level (Lewis, Abhayaratne and Blott, 2015). Registration data has been used to generate the estimated effective population size (EEPS) for many UK dog breeds, and these are available on the Kennel Club UK website (The Kennel Club Limited, 2016).

1.8.9 Intraindividual synchrony of telomere length

While wide inter-individual variations in telomere length exist, intra-individual synchrony in telomere length among cells from different tissues has been demonstrated in several studies across species and ages. Intra-individual synchrony in telomere length was observed in human newborns among leukocytes, umbilical artery cells and skin cells (Okuda *et al.*, 2002), in 11 tissues from fetuses aged 15 to 19 weeks (Youngren *et al.*, 1998), in cerebral cortex, myocardium, liver and renal cortex of human autopsy cases which were neonates to over 100 years old (Takubo *et al.*, 2002), in skeletal muscle, fat, leukocytes and skin of rhesus macaques (Smith Jr *et al.*, 2011) and in leukocytes, fat and skeletal muscle of 83 dogs aged 4 to 42 months (Benetos *et al.*, 2011).

1.8.10 Longitudinal measurements

As well as telomere length varying between individuals, the rate of telomere attrition is also dependent on the individual. In addition, the rate of attrition varies across the lifespan within individuals. Telomere length change in leukocytes has been demonstrated to vary with age, with there being a faster rate of loss early in life during growth and development, followed by a slow in the dynamic later in life. This may be explained by that, in humans, haematopoietic stem cells replicate around 17 times in the first year of life, roughly 2.5 times a year between

ages 3 and 13, and only 0.6 times a year in adults (Sidorov *et al.*, 2009). For example, Zeichner *et al.* (1999) demonstrated that in 9 different infants the rate of loss of LTL was 270 base pairs per year, roughly 4 times that of human adults. In addition, this was demonstrated in lymphocytes and granulocytes from cats. Samples were taken at 2 week intervals in newborn kittens, a 2 year old and 10 year old cats. Telomere shortening was most rapid in the newborn kittens (Brümmendorf *et al.*, 2002).

Baseline telomere length in longitudinal studies can also affect subsequent measures in individuals. Telomere attrition rate has been shown to be inversely correlated with telomere length at baseline (e.g. Nordfjäll *et al.*, 2009), with those with the longest initial telomere lengths undergoing a larger attrition. Verhulst *et al.* (2013) investigated whether this pattern stemmed from a statistical artefact, namely regression to the mean. Regression to the mean (RTM) occurs where repeated measures are made on the same subject. Simply put, if an observation is made which is relatively very high or low, it is likely to be followed by an observation closer to the subject's true mean (Barnett, Pols and Dobson, 2005). Verhulst *et al.* found that the rate of telomere attrition was based on the first LTL measure, but correcting for RTM reduced the slope of the relationship by 57%.

1.8.11 **Telomere length in minimally proliferative tissues**

Telomere length in cells of minimally proliferative or post mitotic tissues have been shown to be longer than those of highly proliferative cells in several species (e.g. Takubo *et al.*, 2002; Gardner *et al.*, 2007; Smith Jr *et al.*, 2011). It has been hypothesised that by comparing telomere length in post mitotic tissues, such as those that form the brain, heart and skeletal muscle (Campisi and d'Adda di Fagagna, 2007), to telomere length in a highly proliferative tissue in an individual, this could provide information on telomere length dynamics over the individual's life course. This model has been applied to macaques (Smith Jr *et al.*, 2011), humans (Daniali *et al.*, 2013) and dogs (Benetos *et al.*, 2011).

In 71 rhesus macaques, skeletal muscle telomere length was not related to age, but the difference between leukocyte and muscle telomere lengths was significantly related to the age of the individual. The difference in telomere length between the two tissues increased with age (Smith Jr *et al.*, 2011). Whereas, in the study using human samples, in 87 individuals aged 19 to 77, while telomere length was longest in skeletal muscle and shortest in leukocytes (telomere lengths of skin and subcutaneous fat cells were also measured), the difference between leukocyte and skeletal muscle was not related to age. In addition, they found a similar level of decline in telomere length in all tissues with age (Daniali *et al.*, 2013).

The Benetos *et al* (2011) study involved 83 dogs aged 4 to 42 months of a wide range of breeds. 58% were female. The dogs underwent a neutering surgical procedure, and samples of blood, subcutaneous fat and skeletal muscle (cremaster muscle in the males, rectus abdominis muscle in the females) were collected. Telomere length was measured in the 3 tissues by TRF analysis (see Section 4.3.1 for details of this method). Telomere length was significantly shorter in leukocytes than skeletal muscle and fat, but it was not significantly different in fat and skeletal muscle samples. Telomere lengths varied by approximately 6kb within individuals, but were highly synchronised within individuals (i.e. those with long skeletal muscle telomere length also had long leukocyte and fat telomere lengths and vice versa). Skeletal muscle and fat telomere lengths were not significantly related to age, but LTL was significantly negatively related to dog age, with age explaining 6% of the variation in LTL. The difference between leukocyte and muscle telomere lengths by age was then examined. As can be seen in Figure 1.4A, the difference significantly increased with age. The data are displayed as leukocyte minus skeletal muscle telomere length on the y axis to generate an equivalent graph to the single tissue telomere length by age plots. Age explained 43% of the variation in leukocyte minus skeletal muscle telomere length. Repeating this analysis using fat in place of skeletal muscle, resulted in age only explaining 14.2% of the variation in telomere length difference. In

addition, the rate of change between skeletal muscle and leukocyte telomere lengths with age was examined (Figure 1.4B). The relationship showed a faster rate of change in telomere length between the tissues at younger ages.

There has been criticism about this model as skeletal muscle is capable of some repair and regeneration, which may impact telomere length. However, this repair is undertaken by satellite cells, which are distinct from the muscle cells themselves (Dellavalle *et al.*, 2007). In addition, some studies have found telomere shortening in skeletal muscle. For example, in male CAST/Ei mice, oxidative stress resulted in significant telomere shortening in isolated muscle fibres (Ludlow *et al.*, 2014). Venturelli *et al.* (2014) measured telomere length in arm and leg skeletal muscle in young, old mobile and old immobile human subjects. They found no relationship between age and arm skeletal muscle telomere length. However, leg skeletal muscle telomere length was negatively associated with age. Free radical concentration was highest in leg muscle of the old immobile group, and free radical concentration was significantly negatively associated with age.

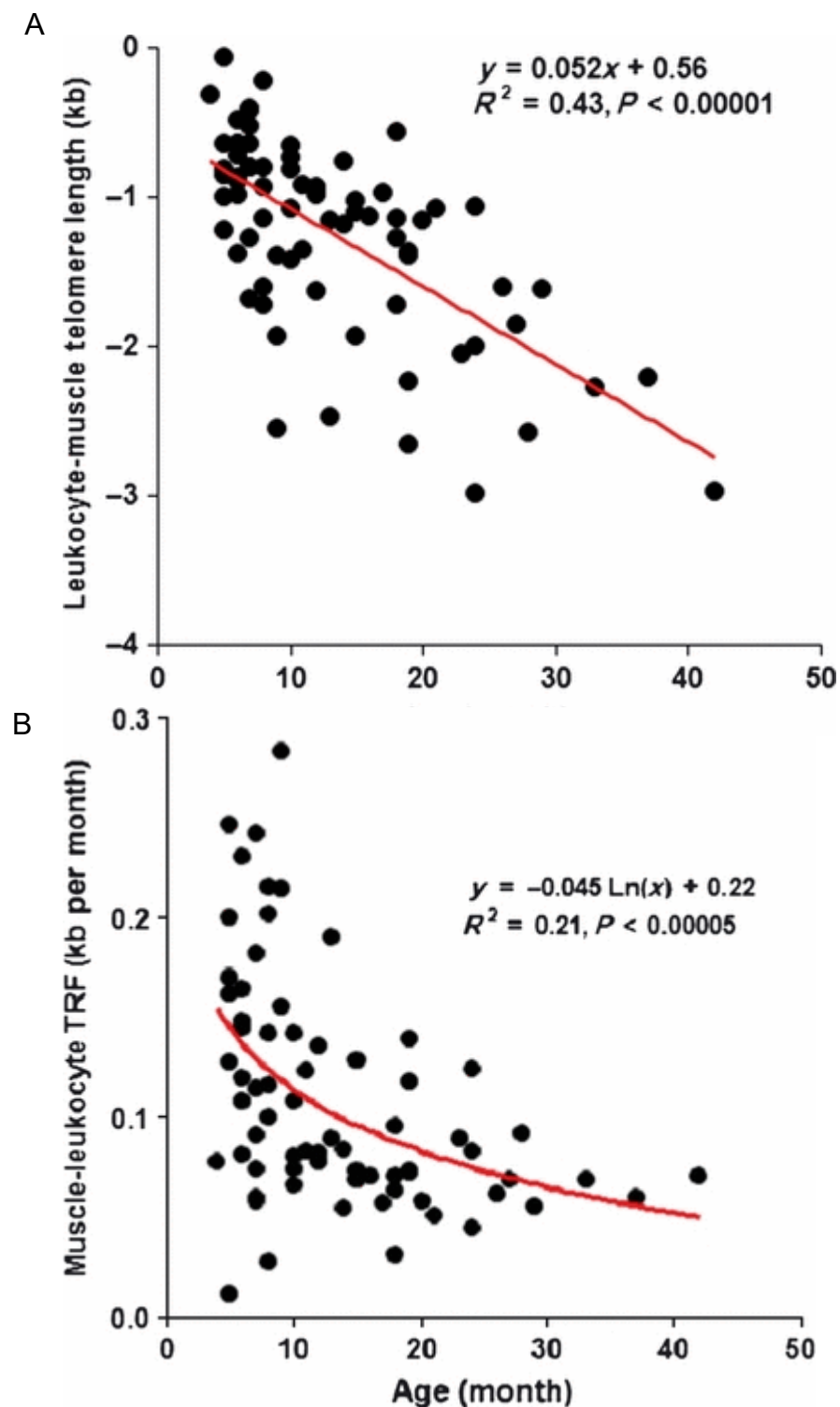


Figure 1.4 Difference in tissue telomere length by age

A significant negative association was observed between leukocyte minus muscle TRF and age in months (A), and a negative curvilinear relationship was observed between muscle minus leukocyte TRF divided by age in months and age (B). The plot in B displays the slowing in rate of change between TRF in the two tissues with age. Copied with permission from Benetos *et al.* (2011)

1.8.12 **Biochemical and haematological measures and telomere length**

1.8.12.1 **Renal function and electrolytes**

Blood sodium, potassium, chloride, calcium, phosphate, urea and creatinine concentrations are routinely measured in dogs attending the University of Glasgow Small Animal Hospital. Estimated glomerular filtration rate (eGFR) is regularly used as a means of assessing renal function in human medicine, with a smaller eGFR indicating poorer renal function level (Cockcroft and Gault, 1976). The majority of studies examining renal function and telomere length use this measure. Raschenberger *et al* (2013) found a significant positive correlation between LTL and eGFR, whereas Huzen *et al* (2014) found no correlation. In addition, Melk *et al.* (2000) found no association between GFR and telomere length measured in human kidney samples. Neuner *et al.* (2015) and Huzen *et al.* (2014) found no significant correlation between LTL and creatinine concentration, which was also the case for a study by Maeda *et al* (2011), who in addition found no correlation between sodium or potassium concentration and LTL.

While no information could be found regarding phosphate levels and telomere length, it has been shown that there is a strong negative correlation between species average serum phosphate and life expectancy in a wide range of mammals (Kuro-o, 2010).

1.8.12.2 **Liver function**

The liver enzymes routinely measured in dog blood samples at the Small Animal Hospital are alkaline phosphatase (ALKP), aspartate transaminase (AST), alanine transaminase (ALT) and gamma-glutamyl transaminase (GGT). In a group of human patients with hepatitis C, ALT and AST were significantly negatively correlated with CD4⁺ and CD8⁺ telomere lengths (LTL) when the patients were not receiving pegylated-interferon-alpha plus ribavirin treatment, but no correlation was found in a second group of patients who did receive the treatment (O'Bryan *et al.*,

2011). In a group of female human patients with hypertension, there was no significant correlation between LTL and AST, ALT or GGT levels (Maeda *et al.*, 2011).

1.8.12.3 **Albumin and globulin**

No significant correlation was found between LTL and albumin in a group of 500 male Caucasians (Raschenberger *et al.*, 2013), whereas in a group of female patients there was a significant positive correlation LTL and both albumin and albumin:globulin ratio, and a significant negative correlation between LTL and globulin (Maeda *et al.*, 2011).

1.8.12.4 **Cholesterol, triglycerides and glucose**

In the same study as previously mentioned, Maeda *et al.* (2011) elicited no significant correlation between LTL and LDL or HDL cholesterol, nor with triglycerides or fasting blood glucose. In the study of 500 male Caucasians, there was a significant positive correlation between LTL and HDL-cholesterol, but no correlation between LTL and total cholesterol. There was a significant negative correlation with glucose levels (Raschenberger *et al.*, 2013). In a group of 343 adults, cholesterol and glucose were significantly negatively correlated with LTL, but not with triglycerides (Neuner *et al.*, 2015). In a further group of 8074 adults, glucose, cholesterol and also triglycerides were significantly negatively correlated with LTL (Huzen *et al.*, 2014). In contrast, in a cohort study of 382 Glaswegian adults, there was a significant positive correlation between LTL and blood cholesterol level (Shiels *et al.*, 2011).

1.8.12.5 **Haemoglobin and erythrocytes**

In a group of over 3000 adults, there was a significant negative correlation found between LTL and haemoglobin concentration, mean cell volume (MCV) and red cell distribution width (RDW) (Kozlitina and Garcia, 2012). De Meyer *et al.* (2008) also

observed a negative correlation between LTL and MCV, but a significant positive correlation between LTL and red blood cell count (RBC). Neuner *et al.* (2015) noted a significant positive correlation between LTL and RBC as well, in addition, they observed a significant positive correlation between LTL and haemoglobin concentration and haematocrit percentage. No correlation was found between LTL and MCV, mean cell haemoglobin (MCH) or mean cell haemoglobin concentration (MCHC). Meyer *et al.* (2016) adjusted the haematological parameters for age and BMI, in their study using samples from humans aged 60 to 84 years, and then found a significant negative correlation between both MCH and MCHC and LTL, but in men only. RBC was found not to correlate with LTL in a group of laboratory mice (Hao *et al.*, 2005).

1.8.12.6 Leukocytes

Multiple studies have observed a significant positive correlation between LTL and leukocyte count (Hao *et al.*, 2005; Gutmajster *et al.*, 2013; Neuner *et al.*, 2015). When the white cell differential was examined by Gutmajster *et al.* (2013), the only type of white cell which significantly correlated with LTL was basophils. In contrast, several studies found no correlation between LTL and white cell count (Satoh *et al.*, 1996; De Meyer *et al.*, 2008; Maeda *et al.*, 2011; Kozlitina and Garcia, 2012; Meyer *et al.*, 2016).

It has been observed that the rate of telomere shortening varies between types of leukocyte. Brümmendorf *et al.* (2002) examined feline lymphocytes and granulocytes, measuring telomere length by flow-FISH. They found age-dependent telomere attrition was faster in lymphocytes than in granulocytes. This finding was also seen in human samples by Rufer *et al.* (1999). In contrast, in another study in humans, across a range of ages telomere length declined at a similar rate in neutrophils and T lymphocytes. In addition, telomere lengths in T cells were on average 372 base pairs longer than in neutrophils (Robertson *et al.*, 2000). This difference in length was similar to that found by Terasaki *et al.* (2002), who observed an average 310 base pair difference. They also noted a similar attrition

rate in telomere length between the two cell populations. Differences in lymphocyte subpopulations have also been observed (e.g. Lin *et al.*, 2010), however data on lymphocyte subpopulation levels will not be available in the dogs in this thesis, so the details will not be given here.

With the differences in telomere length of leukocyte cell populations in mind, the neutrophil to lymphocyte and lymphocyte to monocyte ratios was examined against telomere length in this thesis. In addition to the differences noted in telomere length between the cell types in previous studies, both neutrophil to lymphocyte ratio and lymphocyte to monocyte ratios have been found in multiple human studies to predict cancer survival rate (e.g. Walsh *et al.*, 2005; Porrata *et al.*, 2012; Proctor *et al.*, 2012). A recent study in dogs showed that neutrophil to lymphocyte ratio was significantly increased in dogs with soft tissue sarcoma compared to benign soft tissue tumours (M. J. Macfarlane *et al.*, 2016).

1.8.12.7 Platelets

Significant positive correlations were observed between LTL and platelet count by Hao *et al.* (2005) and Kozlitina and Garcia (2012). No correlation was observed between LTL and platelet count in two further studies (Maeda *et al.*, 2011; Gutmajster *et al.*, 2013).

1.8.13 Psychological stress and telomere length

Psychological stress has been found to be associated with increased levels of oxidative stress in multiple species. For example, increased levels of oxidative stress markers were found in mice subjected to whisker removal and male Sprague-Dawley rats exposed to “conditioned emotional stimuli” by being housed next to rats being intermittently electrically shocked (Adachi, Kawamura and Takemoto, 1993; Wang *et al.*, 2007). However, no significant differences were found in oxidative stress or inflammation markers between humans diagnosed with major depressive disorder and controls. Yet, the duration of the illness was significantly inversely related to LTL, as was oxidative stress markers in both controls and cases (Wolkowitz *et al.*, 2011).

In humans, significantly shorter LTLs have been demonstrated in association with psychological stressors such as intimate partner violence (Humphreys *et al.*, 2012), being diagnosed with a mood disorder (Simon *et al.*, 2006), and being a caregiver to a chronically ill child (Epel *et al.*, 2004). In addition, in the Epel *et al.* study, a numerated level of perceived stress and years of caregiving for a chronically ill child were both significantly positively related to oxidative stress index. However, Parks *et al.* (2009) found no significant association between perceived stress level and LTL in women aged 35 to 74. Yet, there was a significant relationship with LTL in those with both higher perceived stress and above average urine catecholamine concentration, indicating there was an interaction between biological and psychological factors at play.

Psychological stressors have also been found to be related to telomere length in other species. For example, significantly shorter telomere lengths were found in singly-housed than pair-housed African grey parrots, a highly sociable bird (Aydinonat *et al.*, 2014). Offspring of wild caught house mice that were kept in crowded conditions by not removing subsequently produced offspring had significantly shorter telomere length than controls kept in non-crowded conditions (Kotrschal, Ilmonen and Penn, 2007). No studies could be found regarding

psychological factors and telomere length in dogs. However, in a study examining relationships between anxiety, fear and health and lifespan in dogs, stranger directed fear was related to shorter lifespan (Dreschel, 2010).

1.8.14 Socioeconomic status and telomere length

1.8.14.1 Examples of the association between socioeconomic status and telomere length

While measures such as employment status may not be measurable in dogs, there have been studies looking at the effect of parental socioeconomic factors on child telomere length and the effects found in children may potentially be similar in dogs. The West of Scotland Twenty-07 Study is a cohort study examining the social factors which potentially contribute to health inequalities, with individuals divided into group based on if they were born in the 1970s, 1950s or 1930s (Benzeval *et al.*, 2009). The study investigated if social factors from when the participants were age 15 impacted adult LTL. In the 1970s cohort only, there was a significant positive association between parental social class and adult LTL. For the 1970s and 1950s groups, if the family owned a car when they were 15 they had a significantly longer adult telomere length (Robertson *et al.*, 2012). Theall *et al.*, (2013) found that children living in neighbourhoods perceived to have high levels of disorder had significantly shorter salivary telomere length than those who did not live in such a neighbourhood. Factors contributing to a label of “high disorder” included presence of strewn garbage, broken glass, vacant buildings and graffiti. Mitchell *et al.* (2014) examined saliva telomere length and social factors in forty 9 year old boys who participated in the Fragile Families and Child Wellbeing Study. Living in a disadvantaged environment resulted in significantly shorter telomere length, while increased parental income and having a mother with at least some postsecondary education (versus less than a high school education) resulted in significantly longer telomere length.

In contrast, other studies did not find a link between family socioeconomic factors and child telomere length. In the Newcastle Thousand Families Study, adult LTL was not associated with social class at birth (Adams *et al.*, 2007). Asok *et al.* (2013) found no association between buccal cell telomere length in children and household income.

1.8.14.2 **Scottish Index of Multiple Deprivation**

The Scottish Index of Multiple Deprivation (SIMD) is a measure produced by the Scottish government which ranks areas of Scotland from most deprived (rank 1) to least deprived (rank 6,505). It is based on a combination of measures of income, employment, health, education, skills and training, housing, geographic access and crime (The Scottish Government, 2012). The Scottish Neighbourhood Statistics government website, www.sns.gov.uk, can be used to obtain measures of deprivation at a postcode level. In addition, in 2013, in the 20% most deprived areas of Scotland as measured by SIMD, 39% of people smoked, compared to 11% of people in the 20% least deprived (The Scottish Government, 2015a).

1.9 **Alternative tissues to blood in telomere length studies**

1.9.1 **Why an alternative to blood is required**

To take a blood sample specifically for a study from a dog, a Home Office licence is required. Dogs, along with primates, cats and equidae, have additional protection over other vertebrates (Home Office, 2015) making a project licence harder to obtain in these animals. In addition, there are risks associated with blood sampling. These risks are uncommon, but include injuries to the limb or neck, stress to the patient, haematoma at the puncture site, thrombophlebitis, bite injuries to the team performing the procedure and failing to obtain a sufficient sample (British Small Animal Veterinary Association, 2010; Judah, 2014). Clipping fur to expose the vein may lead to dermatitis, so called “clipper rash” (Mason, 2004).

1.9.2 Potential alternative tissues to blood for telomere studies

For a tissue to be a viable alternative to blood for the needs of this study, it needed to be able to be obtained without the need for a Home Office licence, to be acceptable to owners, the DNA extracted needed to be of sufficient quantity and quality for telomere length analysis (see Chapter 4) and preferably to have been used in previous studies to successfully demonstrate age-related telomere changes.

1.9.2.1 Buccal mucosa and saliva

Buccal and saliva samples have previously been used in human telomere studies (e.g. Broberg *et al.*, 2005). The different methods of obtaining oral DNA include: oral rinse, whole-saliva, saliva sponge, buccal swab and cytobrush (Rogers *et al.*, 2007; Mitsouras and Faulhaber, 2009). Oral rinse and whole-saliva methods would not be possible to implement in our study, as oral rinse involves the participant “swishing” with mouthwash and whole saliva requires the participant to deposit saliva into a cup (Rogers *et al.*, 2007), both of which would be rather difficult to get a dog to do.

Cytobrushes were used to collect DNA from 3 dogs by Oberbauer *et al.* (2003). The resulting DNA amplified uniformly in PCR of 8 canine microsatellite markers, demonstrating the quality of DNA extracted. Each swab gave enough DNA for at least 200 PCR reactions. In addition, they found owners were more willing to include their dogs in a study when buccal samples were requested than when blood was asked for. Mitsouras and Faulhaber (2009) compared DNA yield and quality between saliva and buccal swabs in 15 dogs, 3 of whom also had blood samples taken for DNA extraction. To obtain the saliva, they used a specially designed sponge which is marketed as being for use in animals. They found DNA yield from saliva was higher than from blood or buccal samples. Buccal swabs produced, on

average, poorer quality DNA as measured by spectrophotometry, compared to saliva samples. Buccal samples have previously been used to measure canine telomere length, however the author did not state the method of sampling and did not report if age correlated with telomere length or not (Cadile *et al.*, 2007).

One disadvantage of using oral samples is the presence of bacteria in the mouth, which would be co-extracted with the dog's cells. When human saliva and buccal swab samples were tested, the percentage of human DNA content ranged from 5.7 to 100% of the total DNA. The buccal swabs had a higher proportion of human DNA than the saliva samples. In dogs, this is of particular concern given that when 162 dogs were examined at post mortem, 63.6% had periodontitis and this rose to 80% in dogs aged 6 or over (Hamp *et al.*, 1984). The plaque seen in this disease consists mainly of bacteria in a biofilm (Harvey, 1998).

1.9.2.2 Nails and fur

Nails and fur are another potential minimally invasive source of DNA. However, in the case of nails, some dogs dislike and resist having their feet handled, others may previously have had their nails cut too short, resulting in pain and bleeding (Judah, 2014). In addition, many dogs would not have long enough claws to collect sufficient clippings. Telomerase is present in high levels in hair follicles (Ramirez *et al.*, 1997) and no information could be found on telomerase activity, or telomere length, in hair itself or in nails. Oberbauer *et al.* (2003) performed DNA extractions on dog toenail clippings and found the resulting DNA provided variable results when used in PCR, so the DNA quality was poor.

1.9.2.3 Buccal mucosa and saliva telomere length correlation with age

Some studies were able to demonstrate a negative correlation between age and telomere length using oral DNA samples, others not. All studies found on a literature search were on human samples only. Hou *et al.* (2013) measured

telomere length in samples collected from 1,234 individuals by an oral-rinse method, and found a significant negative correlation between mean telomere length and age group. Shalev *et al.* (2013) measured telomere length in buccal swab samples collected from children at two time points: at age 5 and age 10. The mean telomere length of all children at age 10 was significantly shorter than the mean telomere length at age 5. A significant negative correlation was also found between age and saliva telomere length by Lahnert (2005), but the author did not state the method of saliva collection. Broberg *et al.* (2005) measured telomere length in samples collected by oral rinse by qPCR in a group of patients with bladder cancer and a control group. An inverse relationship was found between age and telomere length only in the bladder cancer cases, not the controls. A similar result was found by Thomas, O' Callaghan and Fenech (2008), who measured telomere length in samples collected using a small headed toothbrush against the inside of the cheek. A significant negative correlation between age and telomere length was found in individuals with Alzheimer's disease, but not the study control group.

1.10 Telomerase reverse transcriptase

1.10.1 TERT function

Telomerase is an enzyme found universally in eukaryotes. It is a ribonucleoprotein which depends on two parts: telomerase reverse transcriptase (TERT) and telomerase RNA (TERC), in order to synthesise telomere repeats and lengthen telomeres (Liu *et al.*, 2000; Li and Liu, 2002). The TERC sequence is complementary to the telomere repeat sequence of TTAGGG.

TERT is required for development. TERT deficient embryonic mouse stem cells exhibited genome instability, aneuploidy and telomeric fusions (Liu *et al.*, 2000). TERT and TERC are both expressed in human embryonic stem cells, but then are downregulated during the differentiation process (Yang *et al.*, 2008).

In non-embryonic cells, TERT expression is associated with cell survival and proliferation. In a study of TERT knockout zebrafish, impaired cell proliferation was present, as demonstrated by the S-phase marker PCNA (proliferating cell nuclear antigen) and a lack of spermatogenesis in testes. In addition, an increase in cell senescence marker β -galactosidase in testes, kidney marrow and gut was seen relative to wild type fish. The lack of TERT and therefore telomerase resulted in a p53 mediated DNA damage dependent pathway being activated, leading to cell cycle arrest and apoptosis. The knockout fish died prematurely compared to wild-type fish (Henriques *et al.*, 2013). MYC proto-oncogene encodes the c-MYC transcription factor and is involved in cell proliferation and differentiation. The TERT promoter contains multiple c-MYC binding sites, and therefore c-MYC can increase telomerase activity through TERT binding (Greenberg *et al.*, 1999; Wu *et al.*, 1999). In addition, *in vitro* TERT is activated by both epidermal growth factor and insulin growth factor-1 (Maida *et al.*, 2002; Wetterau *et al.*, 2003). Furthermore, telomerase activity is regularly found in tumours. In 27 canine mammary gland tumours, 26 had detectable telomerase activity, compared to 4 out of 12 normal mammary tissue samples (Yazawa *et al.*, 2001).

1.10.2 TERT and age

No studies could be found on TERT and age, however the whole telomerase protein has been shown to decline with age in normal human T and B cells (Hiyama *et al.*, 1995; Lin *et al.*, 2015). In addition, when telomerase activity was measured in leukocytes obtained from 124 healthy humans aged 4 to 95, a progressive decline in activity was seen between ages 4 and 39, and those 40 and over either showed stable but very low activity or had no detectable activity (Iwama *et al.*, 1998).

1.10.3 TERT and breed

While it has been suggested that dog breed influences telomere length (Section 1.8.3), breed may not influence TERT. When the TERT gene of 10 dogs each of

four breeds, Shih Tzu, Dachshund, Irish wolfhound and Newfoundland were sequenced, polymorphisms did not segregate by breed, and therefore the authors suggested that polymorphic diversification of TERT preceded breed derivation (McAloney *et al.*, 2014).

1.11 CDKN2A

1.11.1 CDKN2A and the cell cycle

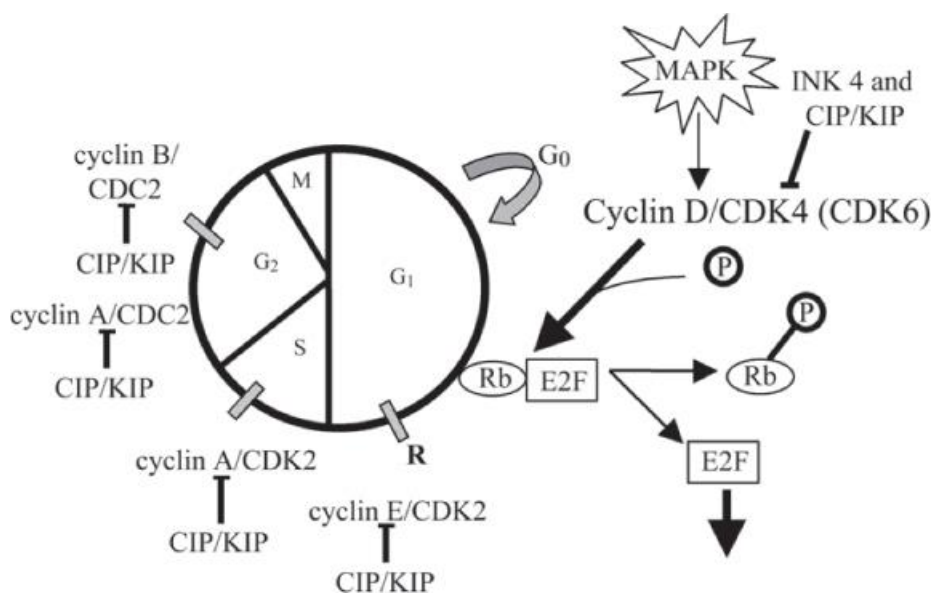


Figure 1.5 Cell cycle

Copied from Harper and Brooks (2005) with permission. The cycle is split into mitosis (M), the two gap phases (G₁/G₂) and synthesis phase (S). 3 checkpoints exist, represented by the shaded bars.

Figure 1.5 shows the stages of the cell cycle, which is split into interphase, consisting of G₁, S and G₂, and mitosis. Progression through the cycle is regulated through cyclin-dependent kinases (CDKs) (Lees, 1995; Vermeulen, Van Bockstaele and Berneman, 2003). Of the nine identified CDKs, five have been demonstrated to be active during the cell cycle: CDK4, CDK6 and CDK2 in G₁, CDK2 in S and CDK1 in M phase. Different cyclins bind to the CDKs at set points in the cycle. One of the ways the CDKs are regulated is the binding of CDK inhibitors, either to a CDK

alone, or to a CDK-cyclin complex. The INK4 (inhibitor of CDK4) family includes p15(INK4b), p16(INK4a), p18(INK4c) and p19(INK4d) (Harper and Brooks, 2005). p16(INK4a) is also known as CDKN2A (cyclin dependent kinase inhibitor 2A) and was examined in this thesis.

When active, CDKs phosphorylate downstream proteins, the most widely studied of which is the product of the retinoblastoma tumour suppressor gene (Rb) (Kato *et al.*, 1993; Morgan, 1995). The activation of cyclin D-CDK4 and cyclin D-CDK6 complexes results in phosphorylation of Rb protein pockets, which is essential for G1 progression. Cells in G1 can enter G0 resting state. Cells in G0 may be quiescent, senescent or postmitotic. Quiescent cells can resume proliferation in response to cellular signals. Both senescent and postmitotic cells have lost the ability to divide (Campisi and d'Adda di Fagagna, 2007; Gewirtz, 2013). As CDKN2A inhibits CDK4, upregulation of this inhibitor prevents cells from progressing past G1. CDKN2A normally has minimal expression in adult tissues, but levels rise when cellular stress occurs and in senescence, but not in quiescent or post mitotic cells (Alcorta *et al.*, 1996; Hara *et al.*, 1996). Ectopic expression of CDKN2A in human lung fibroblasts *in vitro* resulted in cell cycle arrest and senescence (Zhu *et al.*, 1998). In addition, inactivation of CDKN2A *in vitro* human fibroblasts caused a delay in senescence compared to dermal fibroblast controls (Brookes *et al.*, 2004).

1.11.2 CDKN2A, age and morbidity

There is a measurable increase in senescent cells *in vivo* with age (Dimri *et al.*, 1995), and thus an increase in CDKN2A with age. As well as age, increasing CDKN2A levels have been associated reduced organ function and presence of disease. For example, in human kidney tissues from adults aged 21 to 80, CDKN2A expression increased with age and was higher in rejected kidney grafts than healthy kidneys (Chkhotua *et al.*, 2003). In human skin biopsies from groups of ages (0 to 20, 21 to 70, 71 to 95) there was a significant upregulation of CDKN2A in the elderly group (Ressler *et al.*, 2006). In human whole blood, expression of

CDKN2A exponentially increased with donor age, and also was associated with plasma interleukin-6, a marker of human frailty (Y. Liu *et al.*, 2009). Furthermore, when apoptosis was induced in CDKN2A-expressing cells of wild type mice, lifespan was extended, tumorigenesis was delayed and age-related deterioration of several organs was attenuated (Baker *et al.*, 2016).

No studies could be found examining age-related changes in CDKN2A in dogs. However, links have been demonstrated between CDKN2A and canine cancer. For example, CDKN2A expression was absent or reduced compared to normal tissue in 6 of 7 canine melanoma cell lines and 21 of 26 spontaneous canine melanoma tissue samples (Koenig *et al.*, 2002). In addition, cancer associated loci have been found near CDKN2A in 96% of histiocytic sarcoma affected Bernese Mountain Dogs (Shearin *et al.*, 2012).

1.12 Aryl hydrocarbon receptor

The aryl hydrocarbon receptor (AHR) is part of the pathway responsible for the mediation of toxicological effects of selected halogenated and polycyclic aromatic hydrocarbons, which are found in, for example, cigarette smoke and smog. When unbound, the AHR remains in the cytoplasm as part of a complex. Binding of a hydrocarbon ligand results in the release of AHR from the complex, and the AHR then associates with the AHR nuclear translocator protein (ARNT) (Hankinson, 1995). The AHR/ARNT dimer can bind to xenobiotic-responsive elements (XRE) in DNA, which leads to transcription of AHR target genes (Abel and Haarmann-Stemann, 2010).

When a chemical activates the AHR, this leads to upregulation of AHR-mediated genes, which are primarily genes involved in the detoxification of the chemical (Puga, Ma and Marlowe, 2009). Although, one of the genes governed by the AHR-ARNT dimer is the AHR repressor (Ahrr) gene, activation of which leads to negative

feedback of AHR expression (Baba *et al.*, 2001). In addition, depending on the particular ligand which is taken up by the receptor can affect whether the AHR itself is upregulated or downregulated, which can also vary by tissue and by organism. For example, benzo[a]pyrene was observed to downregulate AHR in cultured mouse embryo fibroblasts, but AHR mRNA was increased in the liver of mice exposed to the hydrocarbon 1,4-bis[2-(3,5-dichloropyridyloxy)] benzene (Maglich *et al.*, 2002; Kann *et al.*, 2005).

Two of the target genes of AHR, P4501A1 and P4501A2, are part of the cytochrome P450 family. When substrates such as 2,3,7,8-Tetrachlorodibenzodioxin (TCDD) are metabolised by these enzymes, there is subsequent reactive oxygen metabolite mediated oxidative stress. This was demonstrated by Park, Shigenaga and Ames (1996), who found a marked elevation in excretion of 8-oxoguanine when CYP1A1 was induced in a hepatoma cell line (Hepa1c1c7).

While the AHR is involved in the mediation of hydrocarbons, the levels of AHR are actually affected more by biological factors than chemical ligands. Studies have shown links between AHR and the cell cycle, with changes being associated with ageing and cancer.

When exposed to TCDD, AHR has been demonstrated to form complexes with the retinoblastoma (Rb) protein. AHR appears to act synergistically with Rb to slow cell cycle progression, by repressing E2F-dependent transcription (Puga *et al.*, 2000). Hepatoma cells from AHR-negative mice and human hepatoma cells transfected with AHR siRNA demonstrate a delay between G₁ and S phases of the cell cycle (Ma and Whitlock, 1996; Abdelrahim, Smith and Safe, 2003). In the absence of a ligand, functional AHR plays a role in cell cycle progression, not slowing. Transgenic mice with constitutively active AHR have been seen to develop stomach and liver tumours (Andersson *et al.*, 2002; Moennikes *et al.*,

2004). In addition, AHR was upregulated in canine mast cell tumours, mammary tumours and osteosarcomas compared to control tissues (Giantin *et al.*, 2013).

In *Caenorhabditis elegans*, AHR-deficiency resulted in an increased mean life span compared to wild-type strains (Eckers *et al.*, 2016). In terms of age-related change, AHR mRNA expression significantly decreased in C3H/a male mice at 26 month old of age compared to 3 months olds (Mikhailova, Gulyaeva and Filipenko, 2005). In Sprague-Dawley rats, hepatic and lung AHR protein levels increased rapidly after birth until day 21 of life, then slowly declined with age (Carlstedt-Duke *et al.*, 1979; Gasiewicz, Ness and Rucci, 1984). No studies could be found regarding AHR and age in dogs.

1.13 Sirtuin 1

The sirtuins are a group of proteins found in all organisms, all of which are orthologues of the yeast protein, silent inflammatory regulator 2 (Sir2) (Rine *et al.*, 1979). Seven have been defined in humans, named numerically sirtuin 1 to 7 (Michishita *et al.*, 2005). Sir2 activity is regulated by changes in cellular ratio of nicotinamide adenine dinucleotide (NAD⁺) to the reduced form, NADH, or level of nicotinamide (Blander and Guarente, 2004). The conversion of NAD⁺ to NADH is important in metabolic pathways, with NADH serving as an energy transfer intermediate (Verdin, 2015). NAD⁺ levels reduce with age. In *C. elegans*, reduction of NAD⁺ results in a loss of worm lifespan (Mouchiroud *et al.*, 2013).

Calorie restriction has been demonstrated to increase lifespan in several species, including mice (Weindruch and Walford, 1982), rhesus monkeys (Colman *et al.*, 2009) and dogs, namely Labrador retrievers (Kealy *et al.*, 2002). Transgenic mice that overexpressed sirtuin 1 (SIRT1) displayed similar phenotypes to mice on a calorie-restricted diet. They were leaner than control mice and displayed better glucose control (Bordone *et al.*, 2007). Theories suggest the link between calorie

restriction and increased lifespan is due to a decrease in NADH, which increases the ratio of NAD⁺ to NADH and activates SIRT1 as a result (Lin *et al.*, 2004). In yeast, it was demonstrated that Sir2 alone does not increase lifespan, NAD⁺ is also required (Lin, Defossez and Guarente, 2000).

Sirtuins are involved in several cellular pathways. They act to deacetylate lysines from proteins and peptides, so their effects are wide reaching. For example, DNA damage leads to acetylation of p53, causing p53 to be activated and subsequent cellular growth arrest or apoptosis. SIRT1 was shown to deacetylate p53 *in vitro*, thus preventing apoptosis in the cell (Vaziri *et al.*, 2001; Kume *et al.*, 2006). The ability of SIRT1 to aid in cell survival is also seen in some cancers. In a hepatocellular carcinoma cell line, SIRT1 was demonstrated to be overexpressed compared to normal liver, and interacted with TERT to maintain telomeres. When SIRT1 was silenced, TERT levels reduced and telomere dysfunction was apparent (J. Chen *et al.*, 2011). Although, in an English setter dog, when SIRT1 was measured in samples from 5 different tumours and peripheral blood mononuclear cells (PBMCs), SIRT1 was lower in the tumour samples compared to the PBMCs (Marfe *et al.*, 2012).

SIRT1 decreased with age in mice aorta samples (Donato *et al.*, 2011) and in testis and thymus samples, but not brain (Sasaki *et al.*, 2006). Protein levels of SIRT1 were measured in serum of 250 health human adults. In women, there was a statistically significant pattern with age: an increase from their twenties to thirties, followed by a significant decrease in their forties. However, there was no significant pattern with age in the male subjects (Lee and Yang, 2017).

1.14 Clusterin

1.14.1 Clusterin function

Clusterin was first described in 1983, and was found to be the major glycoprotein in ram rete testis fluid. It was reported to cause clustering of suspensions of rat sertoli cells, mouse testis TM-4 cells and erythrocytes (Blaschuk, Burdzy and Fritz, 1983; Fritz *et al.*, 1983). Clusterin has been found in many species under a variety of functions, often at the time being named by the biological association by which it was recognised. The many alternative names have included SGP-2 (sulphated glycoprotein 2), apoJ (apolipoprotein J) and TRPM-2 (transient receptor cation channel, subfamily M, member 2) (Grassilli *et al.*, 1992; Wong *et al.*, 1993; Jordan-Starck *et al.*, 1994). Despite the many names and functions, clusterin has an estimated 70 to 80% sequence homology between mammals. An inaugural international workshop on the protein agreed on the name clusterin (Wilson and Easterbrook-Smith, 2000), and for simplicity in this thesis all alternative names will be converted to clusterin in the text.

The clusterin (CLU) gene encodes for two types of clusterin protein: an extracellular form (sCLU) and an intracellular form (nCLU). The majority of clusterin protein is in the sCLU form, but in some cell types nCLU occurs in times of apoptosis induction (Trogakos and Gonos, 2009). sCLU has two key functions: firstly, as an apolipoprotein associated with high density lipoprotein (De Silva *et al.*, 1990), and secondly, to stabilise misfolded proteins under a variety of stresses to inhibit protein precipitation (Humphreys *et al.*, 1999; Poon *et al.*, 2000). This second function has resulted in sCLU being considered as a homologue for heat shock proteins (Gudi and Gupta, 1993). Viard *et al.* (1999) exposed a human epidermoid carcinoma cell line (A431) to a variety of stresses: heat shock using temperature-controlled water baths, chemical stress using hydrogen peroxide or hypoxanthine-xanthine oxide, hyperoxia and ultraviolet A. A strong increase in CLU mRNA following these exposures was reported. Anti-sense clusterin clones were then produced. The clones were very sensitive to apoptotic cell death

following exposure to the same stressors, further demonstrating the role of clusterin in cellular stress response. Overexpression of clusterin has repeatedly been shown in *in vitro* studies to increase cell survival after stress exposure (e.g. Dumont *et al.*, 2002). Furthermore, *in vitro* clusterin was found to interfere with Bax activation in mitochondria to prevent apoptosis (Zhang *et al.*, 2005).

Petropoulou *et al.* (2001) demonstrated that in human fibroblasts clusterin mRNA and protein levels were upregulated with cell senescence. They suggested that clusterin did not induce senescence but the increase was a secondary consequence of the senescent phenotype. In addition, contrary to previous reports, clusterin provided no apoptosis protection when the cells were exposed to hydrogen peroxide.

1.14.2 Clusterin and morbidity

Clusterin has been proposed as a biomarker of disease. In dogs presenting at the University of Glasgow Small Animal Hospital for clinical investigation, mRNA levels of clusterin were elevated in cerebrospinal fluid of dogs with degenerative myelopathy and chronic intervertebral disc disease, but not idiopathic epilepsy or meningoencephalitis (Shafie *et al.*, 2014). Clusterin protein levels were raised in sputum of children with asthma compared to healthy controls, and concentration was associated with severity of disease (Sol *et al.*, 2016). In addition, when serum clusterin was measured in serum of 183 humans undergoing coronary angiography, clusterin concentration was significantly higher in patients with significant coronary artery stenosis than those without (Poulakou *et al.*, 2008).

1.14.3 Clusterin and age

In terms of age-related change, clusterin has been demonstrated to increase with age in rat brain (Senut *et al.*, 1992), rat ventral prostate (Bettuzzi *et al.*, 1994), and rat kidney (Laping *et al.*, 1998). In Norway Brown rat epididymis, caput-

corpus clusterin did not change with age, whereas cauda clusterin increased from age 6 to 18 months, then declined at 24 months (Viger and Robaire, 1995). No studies could be found regarding the relationship between clusterin and canine age.

1.15 DNA methylation

1.15.1 Methylation of DNA in the genome

Cytosine can be modified by the covalent attachment of a methyl group to the C5 position of its ring to form 5-methylcytosine (5-mc) (Razin and Riggs, 1980). 5-mc has been found in DNA of animals including in mammals, amphibians and reptiles (Vanyushin *et al.*, 1973). The majority of 5-mc residues are found in the sequence 5'-CG (also known as CpG). There are portions of the genome known as CpG islands where there is a higher frequency of CpG than the rest of the genome. CpG islands are found at the 5' end of all housekeeping genes (Gardiner-Garden and Frommer, 1987). More than 90% of 5-mc residues in the genome lie within CpG islands located in transposable repetitive elements such as Alu elements and LINE-1 (long interspersed nucleotide element-1) sequences (Bollati *et al.*, 2009). 34% of the canine genome is made up of such repetitive elements, which is a lower percentage than in many other mammals including humans (Smit, 1999; Whitelaw and Martin, 2001; Lindblad-Toh *et al.*, 2005). When investigating 5-mc, the percentage of 5-mc residues in the genome may be measured, or as so many of the 5-mc residues are located in repetitive elements, measurement of proportion of 5mc in LINE-1, for example, has been shown to represent the whole genome (Yang *et al.*, 2004).

Methylation is a necessary process for life. When mutation in the enzyme responsible for the reaction transferring the methyl group onto cytosine was introduced into the germline of mice, the result was a lethal phenotype. Embryos were stunted and did not live past mid gestation (Li, Bestor and Jaenisch, 1992). Methylation and changes in methylation status affect gene expression and

function. For example, an increase in 5-mc was observed with T cell differentiation, then methylation decreased slowly with age following terminal differentiation. The increase in methylation as part of differentiation likely served to silence genes (Golbus, Palellan and Richardson, 1990). A further example of methylation affecting genes, is methylation being widely reported to be a key part of X chromosome inactivation (e.g. Allen *et al.*, 1992).

1.15.2 DNA methylation and age

Changes in methylation state occur over time. Loci in CpG have been observed to gain methylation with age, whereas loci not in CpG islands tend to lose methylation with age (Christensen *et al.*, 2009). In mice and humans, the overall 5-mc content in the genome decreases with age (e.g. Wilson *et al.*, 1987; Christensen *et al.*, 2009). One study relating global DNA methylation to age in dogs was found on literature searching. Using an ELISA, percentage global 5-mc was measured in 16 dogs, weighing between 3.2 and 70kg, aged from 2 months to 14 years. %5-mc increased with age and was not related to weight. Reported mean %5-mc was 43.5 in dogs under 9 months and 81.3% in dogs over 8 years, which is surprising as a previous estimate of mammalian DNA %5-mc was between 2 and 7%, depending on species (Razin and Riggs, 1980; Gryzinska *et al.*, 2016).

The changes in 5-mc content in human genome over time have been used to generate an “epigenetic age”, which can then be compared to chronological age with a view to predict risk of age related mortality or morbidity. In a study of leukocyte DNA from 442 human adults, an increase in epigenetic age compared to chronological age was associated with a higher risk of cancer and mortality (Zheng *et al.*, 2016). In a further study of 1334 humans aged between 70 and 90, one standard deviation increase in baseline epigenetic age was linked to a 22% increase in risk of mortality in the 6-year follow-up period (Marioni *et al.*, 2016). This methodology of producing an epigenetic age was very recently replicated in a study of 42 domestic dogs and 62 grey wolves. The epigenetic age was generated using the methylation frequency of 41 CpG islands in dogs and 67 CpG islands in

the wolves. There was a strong positive correlation between epigenetic age and chronological age in both species. A mix of different pure breeds of dog were included in the study. A non-significant positive correlation was seen in the difference between epigenetic age and chronological age with maximum weight of the dog breed, which the authors suggested may have been a significant relationship with a larger sample size (Thompson *et al.*, 2017).

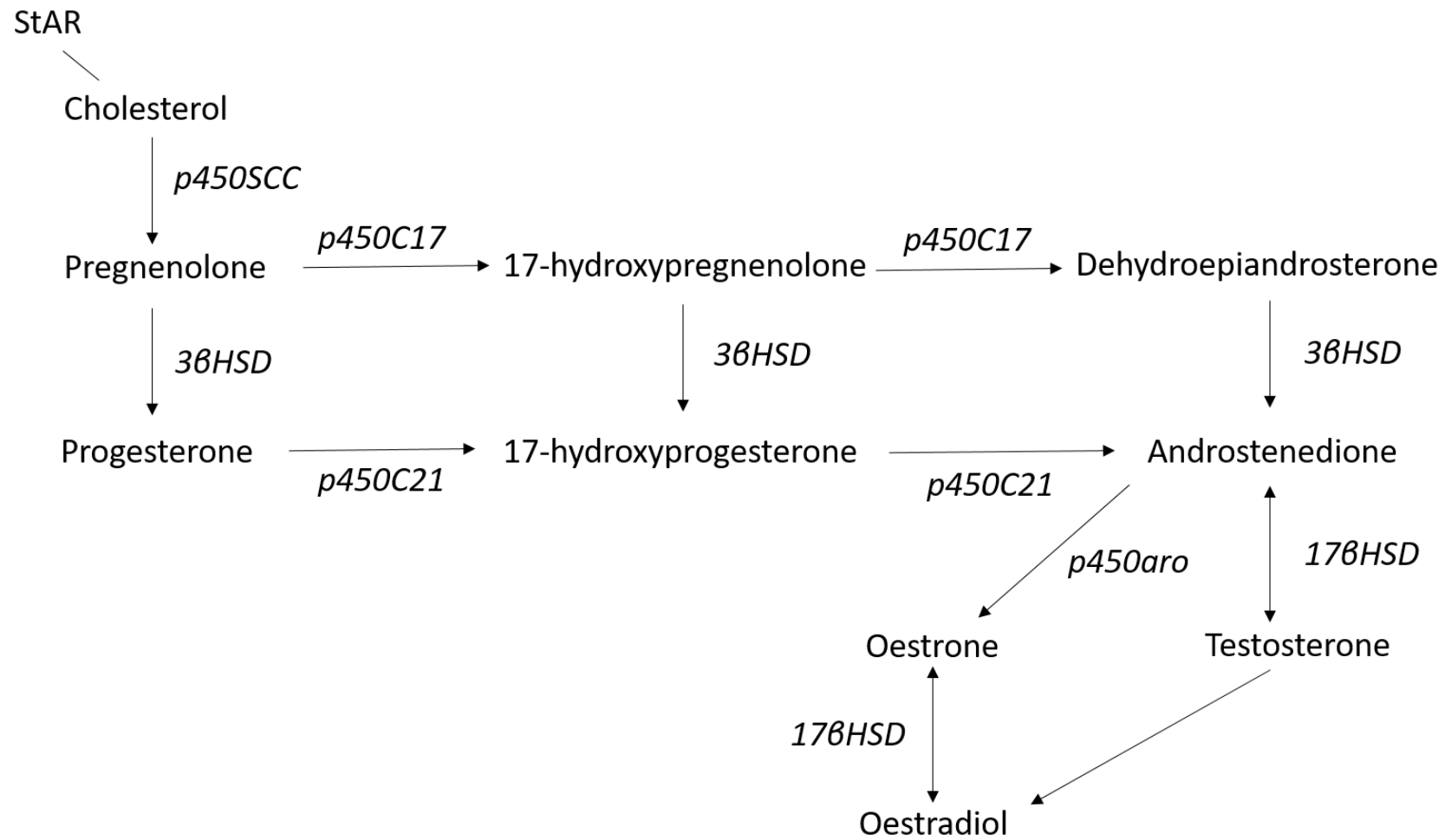


Figure 1.6 Steroidogenic pathway in Leydig cells of the testis
Adapted from Luetjens and Weinbauer (2012)

1.16 Testosterone biosynthesis

Figure 1.6 summarises the steroidogenic pathways in the Leydig cells of the testis. Steroidogenesis is reliant on cholesterol. Cholesterol enters the Leydig cell, most commonly by receptor-mediated endocytosis. Steroidogenic acute regulatory protein (StAR) then plays the essential role of transferring cellular cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane, where the p450 SCC (cholesterol side chain cleavage) enzyme is located (Black *et al.*, 1994; Bauer *et al.*, 2000). Cholesterol is converted to pregnenolone in a single step (Shikita and Hall, 1973). Pregnenolone diffuses across the mitochondrial membrane and is converted to testosterone at the smooth endoplasmic reticulum via several intermediary steps (Simard *et al.*, 2005).

One study regarding change with age of mRNA expression of genes of pathway enzymes in male dogs was published very recently. Three 4-5 month old dogs, seven 6 month old dogs and eight 1 year old dogs of a variety of breeds underwent routine castration. mRNA levels of p450 SCC, p450 C17, 3BHSD (hydroxysteroid dehydrogenase), p450 aro (aromatase) and StAR were measured. p450 SCC, 3BHSD and StAR decreased non-significantly between the 3 age groups, whereas there was a significant decrease in p450 C17. p450 aro increased with age. While several of the genes did not significantly change with age, only 18 dogs with a narrow age range were in the study, and patterns of potential biological interest were apparent in their data plots (Ogawa *et al.*, 2017).

Four further studies in age related changes of the testosterone pathway genes were in Brown Norway rats, using groups aged between 4 and 24 months. StAR and p450 SCC mRNA and protein levels were seen to decrease with age in multiple studies with reductions starting at “middle age” (14/15 months old) (Luo, Chen and Zirkin, 1996, 2001, 2005; Leers-Sucheta, Stocco and Azhar, 1999). Only one of the studies examined 3BHSD and 17BHSD. No reduction in 3BHSD mRNA was seen between 4 months and 22 months of age (17BHSD mRNA

expression was not reported). However, there was a significant decrease in enzyme activity of both 3BHSD and 17BHSD in 22 month old compared to 4 month old rats (Luo, Chen and Zirkin, 1996).

1.17 Overall aims and hypothesis of thesis

The principal key aim of this thesis was to assess the impact of ETS exposure in dogs, with the view of both dog welfare and as a model for human exposures. This was conducted through the measurement of biomarkers of ageing, with the hypothesis that ETS exposure would result in an increased biological age. In order to do this, biomarkers of ageing needed to be measured and examined to explore which would be appropriate for use in dogs and methodologies were developed for their measurement. An objective assessment of ETS exposure was tested in the form of hair nicotine and cotinine concentrations in the dogs, which were then compared to the biomarkers of ageing to examine potential relationships.

Chapter 2: Materials and methods

2.1 Materials

Reagents, kits and solutions used in experiments from all chapters will be detailed in this section, along with consumables and equipment.

2.1.1 General Reagents & Purchased Solutions

For ease of reference, the reagents have been divided into sections according to their use.

Reagents used across multiple experiment types

Reagent	Supplier
Ethanol (molecular biology grade)	VWR, Lutterworth UK
PCR water	Invitrogen, Paisley UK
Phosphate buffered saline (PBS) tablets	Sigma Aldrich, Gillingham UK

Reagents for DNA extraction, RNA extraction and cDNA synthesis

3M Sodium Acetate pH 5.2	Sigma Aldrich, Gillingham UK
Chloroform	Sigma Aldrich, Gillingham UK
dNTP mix	Sigma Aldrich, Gillingham UK
Oligo(dT) ₁₂₋₁₈	Invitrogen, Paisley UK
Phenol	Sigma Aldrich, Gillingham UK
Phenol/Chloroform/IAA	Sigma Aldrich, Gillingham UK
Proteinase K	Macherey Nagel, Düren Germany
RNA <i>later</i> -Ice	Invitrogen, Paisley UK
RNAzap	Applied Biosystems, Paisley UK
Tris EDTA buffer pH 8.0	Sigma Aldrich, Gillingham UK
Trizol	Invitrogen, Paisley UK

Reagents for qPCR & RT-qPCR

Absolute blue low ROX SYBR green qPCR mix	Thermo Scientific, Loughborough UK
DNAzap	Applied Biosystems, Paisley UK
pBR322 plasmid DNA from <i>E.coli</i>	Sigma Aldrich, Gillingham UK
Primers (sequences in results chapters)	Integrated DNA Technologies, Leuven Belgium

Primers arrived lyophilised and were reconstituted to a concentration of $1\mu\text{g}\mu\text{l}^{-1}$ using ddH₂O, then stored in aliquots at -20°C .

Reagents for Southern blotting

Agarose (molecular biology grade)	Invitrogen, Paisley UK
Citric acid	VWR, Lutterworth UK
Ethylenediaminetetraacetic acid (EDTA) disodium salt	VWR, Lutterworth UK
Ethidium bromide 10mg/ml solution	Sigma Aldrich, Gillingham UK
Glacial acetic acid	VWR, Lutterworth UK
Hydrochloric acid 6M	VWR, Lutterworth UK
pH standards: pH 4, 7 and 10	Hanna instruments, Leighton Buzzard UK
Sodium chloride	VWR, Lutterworth UK
Sodium dodecyl sulphate (SDS)	Sigma Aldrich, Gillingham UK
Sodium hydroxide pellets	VWR, Lutterworth UK
Tris base	Sigma Aldrich, Gillingham UK
Tri-Sodium citrate	VWR, Lutterworth UK

Reagents for cell culture

DMEM High glucose media	Invitrogen, Paisley UK
Foetal calf serum	Invitrogen, Paisley UK
Penicillin-Streptomycin	Invitrogen, Paisley UK
Trypsin-EDTA	Invitrogen, Paisley UK

Reagents for cortisol ELISA

HPLC grade methanol	Fisher scientific, Loughborough UK
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2.1.2 Kits

Kit Name	Company	Kit Contents
QIAamp Blood Midi & Maxi kits	Qiagen, Manchester UK	Spin columns, collection tubes, lysis buffer, 2 wash buffers, elution buffer, protease
Nucleospin Blood Mini Kit	Macherey Nagel, Düren Germany	Spin columns, collection tubes, proteinase K, proteinase buffer, lysis buffer, 2 wash buffers, elution buffer
PureLink RNA Mini Kit	Invitrogen, Paisley UK	Spin columns, collection tubes, lysis buffer, 2 wash buffers, RNase-free water, recovery tubes
PureLink DNase Set	Invitrogen, Paisley UK	DNase, reaction buffer, RNase-free water
Superscript II	Invitrogen, Paisley UK	Reverse transcriptase, first-strand buffer, DTT
TeloTAGGG Telomere Length Assay	Roche, Basel Switzerland	Restriction enzymes (<i>Hinf</i> I and <i>Rsa</i> I), digestion buffer, nuclease-free water, control DNA, DIG molecular weight marker, loading buffer, DIG easy hyb granules, telomere probe, washing buffer, maleic acid buffer, blocking buffer, anti-DIG-AP, detection buffer, substrate solution
Cortisol parameter assay kit	R&D systems, Minneapolis USA	Goat anti-mouse microplate, cortisol conjugate, cortisol standard, primary antibody solution, calibrator diluents, wash buffer, colour reagents, stop solution, plate sealers
5-mC DNA ELISA Kit	Cambridge Bioscience, Cambridge UK	Microplate, coating buffer, ELISA buffer, primary antibody, second antibody, HRP developer, negative control, positive control

2.1.3 Solutions & Buffers

Solution/Buffer	Constituents
Tissue lysis buffer	10ml of 5M sodium chloride solution, 10ml of 1M Tris pH 8.0 solution, 25ml of 0.5M pH 8.0 solution and 2.5g SDS solution were added together and the total volume brought to 500ml with deionised water. The buffer was mixed well with a magnetic stirrer and autoclaved prior to use.
6M guanidine hydrochloride	143.31g of guanidine hydrochloride was dissolved in 250ml deionised water to make a 6M solution.
Protein extraction wash solution	10ml of 6M guanidine hydrochloride solution was added to 190ml of 100% ethanol and mixed to make 200ml of solution. This resulted in a concentration of 0.3M guanidine hydrochloride.
1% SDS solution	SDS solution was prepared in a fume hood. 5g of SDS powder was dissolved in 500ml of deionised water to make a 1% solution.
2.5M Sodium hydroxide	50g of sodium hydroxide pellets were dissolved in 500ml of deionised water, resulting in a 2.5M solution.
1M Tris solution, pH 8.0	121.1g of Tris was dissolved in 700ml of deionised water, then the pH adjusted using concentrated hydrochloric acid to pH 8.0, stirring continuously with a magnetic stirrer. The volume of the solution was then made up to 1L with deionised water.
2M Tris solution, pH 7.5	242.2g of Tris was dissolved in 700ml of deionised water, then the pH adjusted using concentrated hydrochloric acid to pH 7.5, stirring continuously with a magnetic stirrer. The volume of the solution was then made up to 1L with deionised water.
5M sodium chloride	292.2g of sodium chloride was dissolved in deionised water to make 1L of solution.

0.5M EDTA solution, pH 8.0	To make 500ml of solution, 93.06g of disodium salt EDTA was added to 400ml deionised water. Sodium hydroxide pellets were slowly added until the pH of the solution reached 8.0, the solution was made up to 500ml with deionised water and mixed well with a magnetic stirrer.
50X TAE buffer	To make 1000ml buffer 242g Tris base, 57.1ml glacial acetic acid and 100ml 0.5M EDTA solution pH 8.0 were combined, then the solution was made up at 1000ml with deionised water and mixed well with a magnetic stirrer.
1X TAE buffer	50X TAE buffer was diluted 1 in 50 to make a 1X working solution, with a concentration of 40mM Tris, 20mM acetic acid and 1mM EDTA.
0.25M Hydrochloric acid	6M hydrochloric acid was diluted 1 in 24 with deionised water to make 0.25M hydrochloric acid solution.
Denaturation solution	To make 500ml of solution, 100ml of 2.5M sodium hydroxide and 150ml of 5M sodium chloride were added to 250ml deionised water and mixed, resulting in a final concentration of 0.5M sodium hydroxide and 1.5M sodium chloride.
Neutralisation solution	300ml of 5M sodium chloride solution were added to 125ml 2M Tris solution pH 7.5 and 75ml deionised water to make 500ml neutralisation solution, resulting in a final concentration of 0.5M Tris and 3M NaCl.
20X SSC	600ml of 5M sodium chloride solution were added to 300ml of 1M sodium citrate pH 7.0 solution and 100ml deionised water to make 1L of 20X SSC, resulting in a final concentration of 3M sodium chloride and 0.3M sodium citrate.
2X SSC	20X SSC was diluted 1 in 10 with deionised water to make 2X SSC.

DIG Easy Hyb Granules	Granules supplied in the TeloTaGGG kit were reconstituted in 64ml of deionised water and the bottle incubated in a 37°C water bath until reconstitution was complete.
Stringent wash buffer I	To make 1L of buffer, 100ml of 20X SSC was added to 100ml of 1% SDS solution and 800ml deionised water, giving a final concentration of 2X SSC and 0.1% SDS.
Stringent wash buffer II	100ml of 2X SSC and 100ml of 1% SDS solution were added to 800ml of deionised water to make 1L of buffer, with a concentration of 0.2X SSC and 0.1% SDS.
1X Washing buffer	The washing buffer is provided in the TeloTaGGG kit at a 10X stock concentration. To create a working concentration buffer, the stock was diluted 1 in 10 with deionised water.
1X Blocking buffer	The blocking buffer is provided in the TeloTaGGG kit at a 10X stock concentration. To create a working concentration buffer, the stock was diluted 1 in 10 with 1X maleic acid buffer.
1X Maleic acid buffer	The maleic acid buffer is provided in the TeloTaGGG kit at a 10X stock concentration. To create a working concentration buffer, the stock was diluted 1 in 10 with deionised water.
1X Detection buffer	The detection buffer is provided in the TeloTaGGG kit at a 10X stock concentration. To create a working concentration buffer, the stock was diluted 1 in 10 with deionised water.
Anti-DIG-AP solution	The anti-DIG-AP antibody was prepared by centrifuging the vial for 5 minutes at 12,000 x g, which reduces background caused by aggregated antibody. 7.5µl of anti-DIG-AP was added to 75ml blocking solution for each blot. The final concentration of antibody was 1 in 10,000 (75mU/ml).

Stripping buffer for Southern blots	To make 500ml of buffer, 40ml of 2.5M NaOH solution, 50ml of 1% SDS solution and 410ml deionised water were mixed together. The final concentrations of reagents in the buffer were 0.2M NaOH and 0.1% SDS.
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2.1.4 Consumables

Item	Supplier
2ml borosilicate glass vial	Wheaton, Millville USA
10cm plastic dishes	Corning, Flintshire UK
15 ml falcon tubes	Greiner, Stonehouse UK
15ml borosilicate test tubes	Fisher Scientific, Loughborough UK
175cm ² Tissue culture flasks	Greiner, Stonehouse UK
20ml scintillation vials	Sigma Aldrich, Gillingham UK
25ml sterile reagent reservoirs	VWR, Lutterworth UK
3mm Whatman filter paper	GE Healthcare Life Sciences, Buckinghamshire UK
50ml falcon tubes	Greiner, Stonehouse UK
96-well PCR plates, semi-skirted, white	Starlab, Milton Keynes UK
Amersham Hybond-N+ blotting membrane	VWR, Lutterworth UK
Cell scrapers	VWR, Lutterworth UK
Cover slips	Chance Proper, Leicester UK
Disposable scalpels	Swann Morton, Sheffield UK
EDTA blood collection tubes	BD Life Sciences, Erembodegem Belgium
Filter pipette tips- 2µl, 20µl, 200µl, 1000µl	Starlab, Milton Keynes UK
Hybridisation bags	Roche, Basel Switzerland
Microamp Optical Cap Strips	Applied Biosystems, Paisley UK
Microcentrifuge tubes: 0.2ml, 1.5ml, 2ml	Greiner, Stonehouse UK and Starlab, Milton Keynes UK
Parafilm	Fisher Scientific, Loughborough UK

Pestle tips	VWR, Lutterworth UK
Pipettes: 10ml, 25ml	Corning, Flintshire UK
Plate seals	Thermo scientific, Paisley UK
qPCR plates clear	Thermo scientific, Paisley UK
RNase free 1.5ml microcentrifuge tubes	VWR, Lutterworth UK
SK-1S DNA Buccal swabs	Isohelix, Kent UK
X-ray film	Thermo scientific, Paisley UK

2.1.5 Equipment

Equipment	Manufacturer
AB 7500 fast qPCR machine	Applied Biosystems, Paisley UK
Agarose gel kit	Bio rad, Hemel Hempstead UK
Balance	Fisher Scientific, Loughborough UK
Benchtop centrifuge	Beckman Coulter, High Wycombe UK
Electronic multichannel pipette	Thermo scientific, Paisley UK
Film cassette	GRI Medical Products, Cave Creek Arizona
Flatbed scanner	Hewlett Packard, California
Haemocytometer	Hawksley, Worthing UK
Heat sealer	TEW, Taipei Taiwan
Incubator	New Brunswick, Hamburg Germany
Kontes electric pestle	Kimble-Chase, Vineland New Jersey
Magnetic stirrer	Stuart scientific, Staffordshire UK
Micro plate spinner	Thermo Scientific, Paisley UK
Microcentrifuge	Technico, Punjab Pakistan
Microcentrifuge with temperature control	Eppendorf, Hamburg Germany
Microplate reader	MTX Lab systems, Virginia USA
Microscope	Leica Microsystems, Milton Keynes UK
Microwave	Sanyo, Osaka Japan
Mini gel kit	Bio rad, Hemel Hempstead UK
MM400 Ball mill	Retsch, Düsseldorf Germany
MxPro 3000P qPCR machine	Stratagene, San Diego, California USA

Nanodrop 1000	ThermoScientific, Paisley UK
pH meter	Fisher scientific, Loughborough UK
Pipette controller	BrandTech Scientific, Essex Connecticut
Pipettes: $\leq 20\mu\text{l}$, $\leq 200\mu\text{l}$, $\leq 1000\mu\text{l}$	Gilson, Luton UK
Power pack	Bio rad, Hemel Hempstead UK
Rocking platform	Stuart scientific, Staffordshire UK
Safety hood	Medical Air Technology, Manchester UK
Shaking incubator	Gallenkamp, London UK
Techne HB-1D Hybridisation oven and bottles	Bibby Scientific, Staffordshire UK
Techne Heat block	Bibby Scientific, Staffordshire UK
Techne Sample concentrator	Bibby Scientific, Staffordshire UK
Thermocycler AB2720	Applied Biosystems, Paisley UK
Timer	Fisher scientific, Paisley UK
Tube roller	Starlab, Milton Keynes UK
UV cross linker	Stratagene, Paisley UK
Vortex	Heidolph Instruments, Schwabach Germany
Water bath	Grant Instruments, Cambridge UK
X ray developer	Xograph Imaging Systems, Gloucestershire UK

2.2 Methods

2.2.1 DNA Extraction from Tissue using Phenol

A ~20mg section was removed from the tissue sample using a disposable scalpel. The section was diced into small pieces on a 10cm plastic dish. A fresh scalpel and dish were used for each sample. The diced sample was placed in a 2ml microcentrifuge tube and 900µl tissue lysis buffer added, along with 100µl proteinase K (10mg/ml). The tube was inverted to mix and incubated for 16 to 72 hours with occasional vigorous mixing, until the tube contents were entirely liquid. 1000µl of phenol was added, the tube was vortexed briefly and then centrifuged at 12,000 x g for 10 minutes at room temperature. Two layers were formed: an upper, aqueous layer and a lower, organic layer. The aqueous layer was removed and put into a new, clean microcentrifuge tube. Care was taken not to disturb the interface or organic phase to avoid transferring these into the new tube. The organic phase and tube were discarded as per the protocol for hazardous waste in the laboratory. An equal volume of phenol was added to the new tube and the process repeated as before. Then this process was repeated twice more, using phenol/chloroform/isoamyl alcohol (25:24:1) in place of phenol. The resulting solution was split into two microcentrifuge tubes. One tenth volume of 3M sodium acetate and two volumes 100% molecular biology grade ethanol were added to precipitate the DNA. The tubes were placed at -20°C overnight. The next day, the tubes were centrifuged at 12,000 x g for 15 minutes at 4°C to form a DNA pellet. The aqueous supernatant was removed and the pellet washed in 70% molecular biology grade ethanol. The pellet was air dried for 20 minutes, then resuspended in 200µl TE solution. The quality and concentration of the DNA was measured as described in section 2.2.5, then stored at -20°C.

2.2.2 DNA Extraction using Columns

Blood, buccal and cell culture samples were DNA extracted using column based kits. The kits used were Macherey Nagel Nucleospin® Blood Mini Kit and Qiagen QIAamp® Blood Midi Kit. The mini size was used for sample volumes up to 200µl

and the midi for sample volumes greater than 200µl up to 2ml. The manufacturers' protocols were followed with minor adjustments.

2.2.2.1 Blood DNA Extraction

Blood samples collected in EDTA tubes were used for the extractions and equilibrated to room temperature prior to starting the protocol. For a proportion of the samples used in Chapter 4, the Qiagen QIAamp midi kit was used to obtain larger amounts of DNA using a single column. For all other blood DNA extractions, the Macherey Nagel kit was used. For brevity, the Macherey Nagel kit protocol will be described only, as the Qiagen kit protocol is largely equivalent bar the use of larger volumes of liquid.

200µl blood was pipetted into a 1.5ml microcentrifuge tube and 25µl Proteinase K added, followed by 200µl of Buffer B3 (kit lysis buffer). The tube was vortexed vigorously for 10-20 seconds to mix the contents. The samples were incubated at 70°C using a heat block for 1 hour. 210µl of molecular biology grade ethanol was added to each sample and the tube vortexed again. The sample was then loaded into a Nucleospin® Blood column placed in a collection tube. The tube was centrifuged at 11,000 x g for 1 minute. The collection tube and flow through were discarded. DNA was now bound to the silica membrane of the column and next the membrane was washed twice. For the first wash, the column was placed in a new collection tube and 500µl of Buffer BW (kit wash buffer) added into the column and then centrifuged for 1 minute at 11,000 x g. Again, the collection tube and flow through were discarded. The column was placed into a new collection tube and 600µl of Buffer B5, the second kit wash buffer, was added and the sample centrifuged at 11,000 x g for 1 minute. The flow through only was discarded, the column placed back into the collection tube and centrifuged for 1 minute at 11,000 x g, to remove residual ethanol. The collection tube was discarded and the column placed in a 1.5ml microcentrifuge tube. 100µl of 70°C preheated Buffer BE (kit elution buffer) was added directly onto the silica membrane. After 1 minute of incubation at room temperature, the tube was centrifuged for 1 minute at 11,000 x g. In order to increase the DNA yield, the elute was placed back onto the column and

the incubation and centrifugation repeated. The DNA was then in the microcentrifuge tube. The quality and quantity were measured and the DNA stored at -20°C .

2.2.2.2 Buccal Swab DNA Extraction

Buccal samples were obtained using Isohelix SK1 swabs. The swab was removed from its tube, placed inside the mouth of the dog and rubbed firmly against the inside of the cheek. The swab was rubbed inside both cheeks for a total of up to one minute. Sampling for one minute increases DNA yield, however, this was sometimes impractical if it was difficult to control the dog or the dog showed signs of aggression. The swab was placed back in the tube and the handle snapped at the provided groove. The swab head with remaining stick was dropped into the tube and the tube sealed with the screw cap provided. The swabs were DNA extracted within 24 hours of sample collection to minimise time for microbial growth.

The swab head was removed from the tube using sterile tweezers and the swab head was cut off from the stick with scissors and placed into a 2ml round bottom microcentrifuge tube. 400 μl of PBS, 20 μl of Proteinase K and 600 μl Buffer B3 (lysis buffer) were added. The tube was vortexed vigorously for 20 seconds and then incubated at 70°C for one hour. From this point, either a phenol-chloroform based protocol or a column based protocol was performed. For the column protocol, firstly, 400 μl of molecular biology grade ethanol was added. The tube was then vortexed. 600 μl of the mixture was loaded into a Nucleospin® column placed in a collection tube and centrifuged for 1 minute at 11,000 x g. The flow through and collection tube were discarded. The column was placed in a new collection tube and the remaining mix loaded into the column, the tube centrifuged for 1 minute at 11,000 x g and the collection tube and flow through discarded. The column was placed in a new collection tube and wash steps and elution procedure were followed as per the blood DNA extraction methodology (section 2.2.2.1).

To follow the phenol protocol, the microcentrifuge tube was centrifuged for 15 minutes at 11,000 x g. The swab was removed and 1000µl phenol was added. The tissue extraction protocol was then followed from the initial addition of phenol step (section 2.2.1).

2.2.2.3 Cultured Cells DNA Extraction

5x10⁶ cells in medium were transferred into a 1.5ml microcentrifuge tube and the tube centrifuged for 5 minutes at 300 x g. The supernatant was removed and the cell pellet resuspended in 200µl PBS. The methodology was then followed as per the blood DNA extraction methodology from the lysis stage (see section 2.2.2.1).

2.2.3 RNA Extraction from Tissue

A section of tissue of approximately 50mg was removed from each sample. The section was placed in an RNase-free 1.5ml microcentrifuge tube and 400µl Trizol added. The section was homogenised using an electric pestle. A further 600µl Trizol was added and the tube was left at room temperature for 5 minutes. 200µl of chloroform was then added and the tube shaken vigorously for 15 seconds. Then followed a 3-minute incubation at room temperature, after which the sample was centrifuged at 12,000 x g for 15 minutes at 4°C. The liquid split into 3 phases: a lower, phenol-chloroform phase, an interphase and an upper aqueous phase containing the RNA. 400µl of the upper phase was transferred into a new RNase-free 1.5ml microcentrifuge tube. The remaining liquid was kept and stored at -80°C, in order to be protein extracted at a later date. 400µl of 70% molecular biology grade ethanol was added to the upper phase tube and the tube was vortexed to mix. The sample was then processed using a PureLink RNA mini kit including an on-column DNase treatment. 600µl of sample was transferred to a Spin Cartridge with a collection tube, the tube was centrifuged for 15 seconds at 12,000 x g and the flow through discarded. The column was replaced into the collection tube and the remaining sample loaded into the column. The tube was centrifuged for 15 seconds at 12,000 x g and the flow through discarded again. 350µl of Wash Buffer I was added to the Spin

Cartridge and centrifuged for 15 seconds at 12,000 x g. The flow through and collection tube were discarded and the Spin Cartridge was inserted into a new collection tube. A DNase treatment on column mix was made using 8µl of 10X DNase I Reaction Buffer, 10µl of 3U/µl DNase and 62µl RNase free water and this was added directly onto the surface of the Spin Cartridge membrane. It was incubated at room temperature for 15 minutes. 350µl of Wash Buffer I was added to the Spin Cartridge and it was centrifuged at 12,000 x g for 15 seconds. The flow through and collection tube were discarded and the Spin Cartridge was inserted into a new collection tube. 500µl of Wash Buffer II with ethanol was added to the Spin Cartridge, centrifuged for 15 seconds at 12,000 x g and the flow through discarded. The Spin Cartridge was reinserted into the collection tube and was washed again with Wash Buffer II twice using the same method. The Spin Cartridge was centrifuged at 12,000 x g for 1 minute, which dried the membrane. The collection tube was discarded and the Spin Cartridge was inserted into a Recovery Tube. 30µl of RNase free water was added to the centre of the Spin Cartridge membrane. It was incubated at room temperature for 1 minute and centrifuged at 12,000 x g for 1 minute. The elute was placed back onto the Spin Cartridge membrane, incubated for another minute and centrifuged for 1 minute at 12,000 x g. The elute contained the RNA sample. The quantity and quality of the RNA was measured, then stored at -80°C.

2.2.4 cDNA Synthesis

cDNA was synthesised from RNA as DNA is required for PCR assays, using the method as follows. For each RNA sample, 2µg of RNA was added to a 0.2ml nuclease-free microcentrifuge tube, along with 1µl oligo(dT)₁₂₋₁₈ and 1µl dNTP mix (10mM each). Sterile, distilled water was added to the contents to make a total volume of 12µl. A “blank” tube was made by adding no RNA but with all other reagents. The tubes were placed in a thermal cycler, heated to 65°C for 5 minutes then immediately placed on ice. The tubes were briefly centrifuged to collect the contents at the bottom of the tube and then 4µl first-strand buffer and 2µl of 0.1M DTT were added. The contents were mixed by pipetting up and down, then the tubes incubated at 42°C for 2 minutes. 1µl of Superscript™II Reverse Transcriptase was added to each tube and then mixed in by pipetting up and down. The tubes were placed at 42°C for 50 minutes, then the reaction was

inactivated by heating at 70°C for 15 minutes. The concentration and quality of cDNA was measured, using the “blank” tube as the blank, and then stored at -20°C.

2.2.5 Spectrophotometry

The quantity and quality of sample post DNA and RNA extractions, cDNA and single stranded DNA oligonucleotides were assessed using a Nanodrop® 1000 spectrophotometer.

2.2.5.1 DNA and RNA quantification

The software uses a modified version of the Beer-Lambert equation for nucleic acid quantification. The correct settings were chosen on the software, such that the extinction coefficient for double-stranded DNA was 50 ng-cm/μl, for single-stranded DNA 33ng-cm/μl and for RNA 40 ng-cm/μl. Absorbance is measured at 230nm, 260nm and 280nm, and 260/280 and 260/230 ratios are calculated by the software. The absorbance values are also corrected relative to a blank. The blank used was the elute buffer for DNA column extractions, TE buffer for DNA phenol-chloroform extractions, RNase-free water for RNA extractions, nuclease-free water for single stranded DNA and, for cDNA, the “blank” tube as described in section 2.2.4. Sample concentration calculations are based on the 260nm absorbance and the extinction coefficient. The 260/280 value is used to assess the purity of the nucleic acid. For our purposes, a value of 1.8 or greater was accepted for DNA and 2.0 or greater for RNA. The 260/230 ratio also assesses purity and a low value indicates the presence of co-purified contaminants, such as phenol. A 260/230 of over 2.0 was seen as ideal, but a value of over 1.8 was accepted. Rules were enforced for these numbers as residual contaminants can lead to overestimation of concentration of nucleic acid and potentially inhibit downstream procedures.

If values for purity measurements in the DNA extractions fell below the required standards, purification steps were repeated until the required standard was met,

or repeat extraction was performed if there was sufficient sample available. To purify DNA, a Macherey Nagel Nucleospin® Blood Mini kit was used. 1 volume of Buffer B3 (kit lysis buffer) and 1 volume molecular biology grade ethanol were added, then vortexed for 20 seconds. This mixture was loaded into a Nucleospin® column and the protocol followed as described in section 2.2.2.1, DNA extraction using columns.

If values for purity of RNA fell beneath the required standards, a repeat extraction was performed using the original tissue.

2.2.6 Quantitative PCR

PCR is a method of generating copies of a specific template spanning from a set of primers, using a repetitive cycle of reactions. When the reaction efficiency is 100%, the amount of product doubles with each cycle, until factors such as depletion of reactants results in a plateauing of product accumulation. As different PCRs can reach plateau at different cycle numbers, traditional “end point” PCR cannot be used for quantification purposes. Quantitative PCR (qPCR, also known as “real time” PCR) enables the user to measure the amount of PCR product as it accumulates, using fluorescent dyes. SYBR green was used in this thesis, which emits fluorescence when bound to double stranded DNA.

A “ ΔR ” or “ dR ” value is calculated by the qPCR machine software, which is the difference between the reaction fluorescence and the background fluorescence. This is plotted against the cycle number, giving the amplification plot. An example plot is shown in Figure 2.1 Example of qPCR amplification plot In the initial cycles, the ΔR remains at baseline until sufficient signal is generated for machine detection (Heid *et al.*, 1996). When the signal generated is significantly greater than the background, the intensity increases in an exponential manner. During this exponential phase, the fluorescent signal is directly proportional to DNA concentration (Raso and Biassoni, 2014). An arbitrary threshold is assigned in the exponential phase, which is the point at which all samples are compared to each other. The number of PCR cycles required to reach this threshold is known as the cycle threshold, or “ C_T ”. The C_T values are directly related to the starting concentration of template and are the

basis for calculations of telomere length (Chapter 4 and 5) or mRNA expression level (Chapter 6). Differences in C_T are used to compare samples. As the reaction is exponential at this point, a difference in C_T of, for instance, 2 is equivalent to a fourfold difference in starting material (Ginzinger, 2002).

There are two key types of qPCR analysis: relative quantitation and standard-curve based “absolute” quantitation. Relative quantitation examines the differences in C_T to the power of 2 (due to doubling of PCR product) and the data generated represents fold difference between items of interest. Both absolute and relative quantitation were explored for telomere experiments. mRNA expression levels were calculated using the relative method, comparing test samples to an internal control sample (Ginzinger, 2002).

As the amount of starting material directly influences C_T , results are usually normalised to a control gene to correct for quantity of DNA added to each plate well (Livak and Schmittgen, 2001). In addition, by including a passive reference dye, such as ROX (6-carboxy-X-rhodamine), in the reaction mix, non-PCR related changes in fluorescence can be accounted for using the settings on the machine software. The ΔR value in the amplification plot can then be normalised to the signal from the passive reference dye (Schuelke, 2000; Wong and Medrano, 2005).

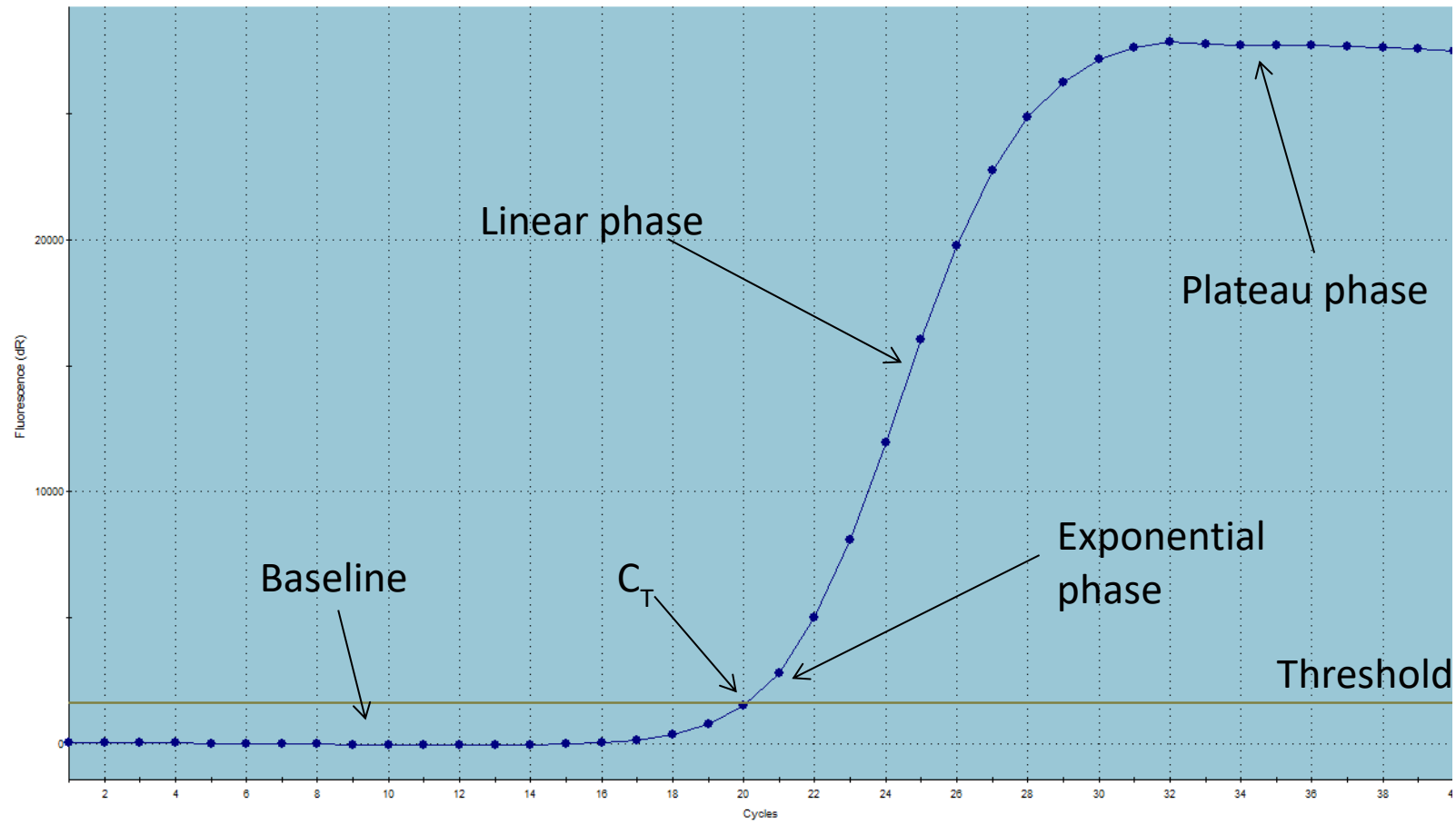


Figure 2.1 Example of qPCR amplification plot

2.2.7 Gel Electrophoresis

Prior to using DNA samples for Southern blotting, the integrity of the DNA was checked by electrophoresis using a 1% agarose gel. The samples were prepared for loading by diluting the DNA to 5ng/ μ l with TE buffer and adding 8 μ l to 2 μ l 5X loading buffer. The samples were then loaded onto the gel and the gel run at 100V for approximately 20 minutes. The DNA bands were visualised using a UV transilluminator and photographed. Figure 2.2 shows an example of a gel photograph of DNA samples tested for integrity prior to Southern blot analysis. If a band did not move in parallel with the others or appeared as a smear, then the corresponding DNA was not used for any further analysis. This is because the use of degraded DNA in Southern blot analysis can lead to inaccurately short telomere measurements.

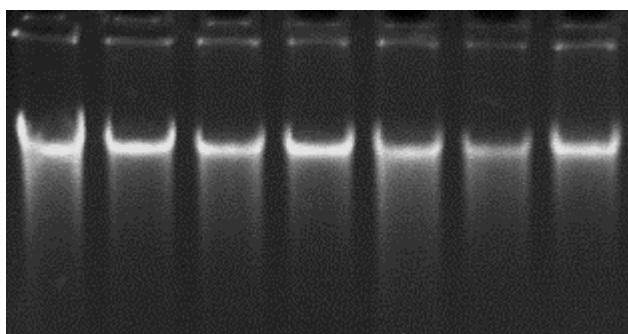


Figure 2.2 Representative gel of genomic DNA
Gel run to check DNA integrity prior to starting Southern blot protocol

2.2.8 Cell Culture

2.2.8.1 Cell Lines

Lines used in this thesis were MDCK cells and HeLa cells. The MDCK cell line was established by S.H. Madin and N.B. Darby in 1958 from the kidney of an apparently normal female Cocker Spaniel (Madin, 1958). The HeLa cell line originated from cervical cancer cells derived in 1951 from a human patient, Henrietta Lacks (Gey, Coffman and Kubicek, 1952). MDCK cells (passage 25) were kindly provided by Prof L Nasir, School of Veterinary Medicine, University of Glasgow, and HeLa cells (passage 26) were kindly provided by Mr A Stevenson, Centre for Virus Research, University of Glasgow. Both lines were cultured in

DMEM High Glucose media supplemented with 10% FCS, 100IU/ml penicillin and 100IU/ml streptomycin. They were kept in 5% CO₂ in a 37°C incubator.

Both HeLa and MDCK were cultured in 10cm dishes and, once confluent, the cell lysate was used for Western blot experiments as described in Appendix 9. MDCK cells were also cultured in 175cm² flasks and aliquots of cells were collected for DNA extraction purposes.

2.2.8.2 Passage of Cells

MDCK cells cultured in 175cm² flasks were split into multiple flasks to generate large numbers of cells for DNA extraction. The cells were grown until 80% confluency was reached, then the media removed and discarded from the flask. The cells were washed with 15ml PBS and then covered with 5ml pre-warmed 37°C trypsin-EDTA. The flask was placed in the incubator for 5 minutes and then examined under a microscope to check the cells had detached. 10ml of pre-warmed medium was dispersed over the cell layer and the cells then transferred into a 15ml tube. The tube was centrifuged at 200 x g for 5 minutes, producing a cell pellet, and the supernatant removed. The cell pellet was resuspended in 5ml of media, 1ml of cell suspension was added to 25ml of fresh media in a new flask and the other 4ml used for DNA extraction, after quantifying the cell concentration as described below.

2.2.8.3 Cell Counting

10µl of cell suspension was introduced into a haemocytometer. The chamber was viewed under a microscope using a 10X objective and the cells in the central gridded square counted. The count was multiplied by 10⁵ to estimate the number of cells per ml. The count was repeated twice and the average between the counts taken.

2.2.9 Environmental Tobacco Smoke Exposure in Pet Dogs Study Outline

2.2.9.1 Initial project outline

The aim was to recruit 40 dogs to the study, 20 from smoking homes and 20 from non-smoking homes. For the sample size power calculation, see section 5.2.1. The dogs were to participate in the study for one year, with 3 monthly appointments. Given that other studies have found differences in telomere length between breeds, it was decided that only one breed would be targeted for recruitment. Staffordshire Bull Terriers are a popular breed in the west of Scotland, however many end up in the care of rescue charities. The Scottish Society for the Prevention of Cruelty to Animals (SSPCA) runs events to help promote the adoption of the breed, due to the numbers in their care and the negative reputation of the behaviour of dogs of this breed. Staffordshire Rescue Scotland is one of many charities that run initiatives to help owners to cover the cost of neutering, as a preventive measure to help “stem the tide” of these dogs ending up in rescue centres. For these reasons, it was decided to focus recruitment on this breed. As there have been studies in humans showing telomere shortening in individuals with chronic illnesses, dogs with longstanding illnesses were excluded from the study. Dogs were required not to have been neutered previously, as neutering and retention of the removed tissues formed part of the study. The dogs could be of any age and either sex, provided they were old enough to undergo neutering surgery.

To establish the environmental tobacco smoke exposure of each dog, a questionnaire was distributed to its owners at 3 monthly intervals and repeat fur samples were taken from the neck of the dog for quantification of nicotine and cotinine concentrations by mass spectrometry.

The dogs had health checks and pre-anaesthetic blood samples taken at a recruitment appointment to ensure they were fit for the study and surgical anaesthesia. The samples were sent to the University of Glasgow Veterinary Diagnostic Service for haematology and biochemistry tests. Results from these

tests were checked by a qualified veterinary surgeon. Discarded blood in the EDTA coated tube following the pre-anaesthetic haematology testing was retained. DNA was extracted from 200µl of the discarded blood as described in section 2.2.2.1. The remainder was centrifuged at 1,500 x g for 10 minutes to separate out the plasma, which was then stored at -20°C. If the dog was deemed fit to partake in the study, it was admitted for a neutering procedure. On the day of the procedure, a buccal swab was collected from the dog. Males underwent a closed prescrotal castration and females an ovariohysterectomy (procedures as described in Fossum 2013). Pre-anaesthetic, acepromazine and opioid analgesia were administered. General anaesthesia was induced with propofol and maintained with isoflurane. Skin sutures used depended on surgeon preference. If non-absorbable sutures were used, an appointment for suture removal was provided 10 to 14 days after the procedure. Meloxicam was administered during the procedure and the owners were provided with a bottle to take home for post-operative oral analgesia. The meloxicam was kindly donated by Boehringer (Ingelheim an Rhein, Germany). If a young male dog was deemed to not be ready for castration (judged by examination by a veterinary surgeon) at the health check appointment, the castration was not performed until its testicles were sufficiently developed.

Surgically removed ovaries and testes were cut into approximately 1cm³ pieces. Pieces from one of the testes or ovaries were then placed in formalin and pieces from the other were snap frozen in liquid nitrogen, then stored at -80°C. Samples were collected from the uterus, in the case of the females, and from cremaster muscle, vas deferens and epididymis, in the case of the males, for DNA extraction purposes. After sampling, the remaining tissue was stored at -80°C. It was planned to take 3 further buccal swabs over the course of the following 12 months, for DNA extraction and telomere length measurement, alongside the owner questionnaire and fur sampling. The aim was to establish whether there was a pattern in telomere length dynamics in the buccal samples with the environmental tobacco smoke exposure across the year. At the end of 12 months, a blood sample was taken as part of a routine annual health screen and sent to the university's veterinary clinical pathology department as

previously. Again, the spare blood after testing was retained, 200µl used for DNA extraction, then plasma separated from the remaining.

2.2.9.2 Revised project outline

5 months after recruiting the first dog to the study, only 8 dogs had been signed up to the study. Therefore, it was decided that the study plan should be changed in order to recruit the further 30 dogs wanted within a shorter time frame. Firstly, the breed criteria were changed. While keeping to one breed was seen as ideal due to literature stating that there are telomere length differences between breeds, only using one breed was limiting for recruitment purposes. The dogs which had been recruited had other issues which may have also had an impact on telomere length, such as obesity and previous psychological stress due to abandonment and rehoming, so focussing on breed seemed short sighted after encountering a few cases. In fact, two of the first 8 dogs recruited were relinquished by their owners during the study period due to issues with aggression in these animals. It was decided that dogs under 5kg and over 50kg should be excluded, as these extremes in size may have had an as yet unknown impact on telomere length. Also non-shedding breeds were excluded, as it was queried whether this coat type may be different in terms of hair nicotine measurements in work previously conducted in pet dogs (Knottenbelt *et al.*, 2012). Finally, it was decided to only use male dogs and exclude females, after the final female booked to attend was seen, for three key reasons. First, because differences in telomere biology have been seen between sexes in both dogs and humans (Okuda *et al.*, 2002; Mayer *et al.*, 2006; Zhu *et al.*, 2011; Fick *et al.*, 2012). Secondly, ovarian pathology was present in all bitches spayed as part of the study and, thirdly, it was not possible to obtain skeletal muscle from the female dogs while working under the Veterinary Surgeons Act (1966). Skeletal muscle was required to replicate the model of telomere dynamics described by Benetos *et al.* (2011).

In addition to the changes in recruitment criteria, the number of appointments over the 12-month study period was reduced due to poor attendance of appointments by owners. The visits were reduced to the pre-operative

screening, the neutering and the 12 month follow up appointments. The owners were asked to respond to e-mails, phone calls or letters (depending on their preference) during the year to establish if the dog's tobacco smoke exposure had remained the same, and if the dog had any illnesses, injuries, psychological trauma or developed any new behavioural problems. It was also hoped that by remaining in contact with the owners that they may be more likely to respond to a request for them to return to the hospital at the 12-month time point. The steps of the revised outline and samples collected at each time point are summarised in Figure 2.3.

2.2.9.3 Recruitment methods

9 of the dogs were recruited with the kind help of Staffordshire Rescue Scotland, who liaised with owners on our behalf prior to the dogs' first visits to the Small Animal Hospital. A request was sent to the local People's Dispensary for Sick Animals (PDSA) hospital for client referrals; however, this yielded no recruits. Information was disseminated to local first opinion veterinary practices in the area, but again this provided no further participants. However, when one practice was informed that a client of theirs was taking part, this did result in one further dog being recruited. One dog belonged to a veterinary student who found out about the study through word-of-mouth. After the breed criteria were changed, an e-mail was sent to all staff and students at the University of Glasgow, which led to many requests to join the study and the remaining dogs needed being signed up.

To thank owners for participating, the neutering and associated aftercare and health check blood tests were provided free of charge. In addition, they were entitled to two doses of worming tablets over the 12-month period for providing up to date information about their dog's health and ETS exposure. At the 12-month appointment (or after depending on the dog's vaccination history) owners were entitled to a free booster vaccination for their dog. The vaccines and wormers were kindly donated by Zoetis (New Jersey, USA).

Recruitment appointment

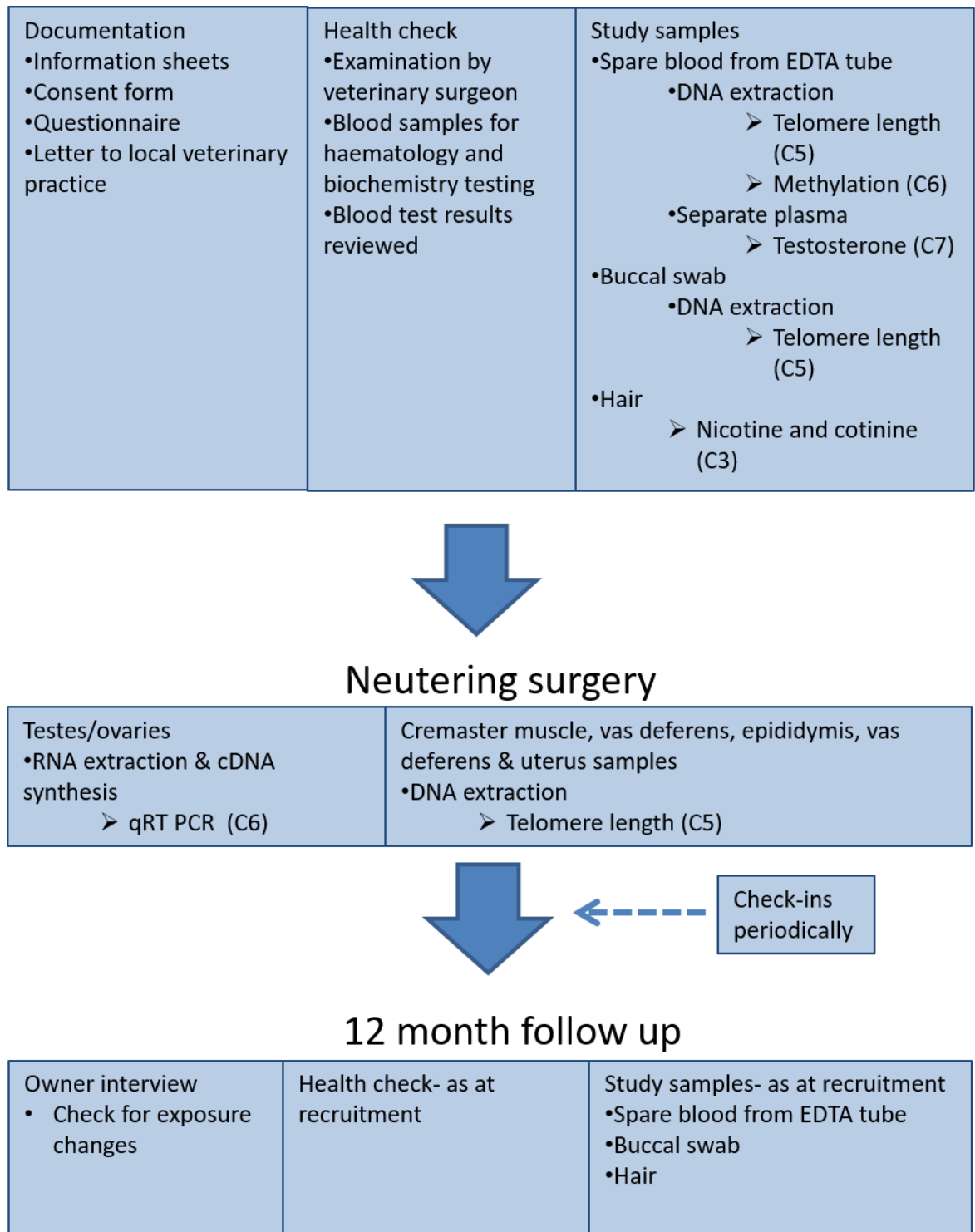


Figure 2.3 Outline of study

Flowchart displaying steps taken at each time point. C indicates the chapter that the relevant data is examined.

The owners were not given any financial payment for their time or travel expense, so the free veterinary services provided were a means of offsetting these factors for the owners and the items provided were of benefit for the health and wellbeing of the dogs.

After the study was completed, the owners were informed of the results generated. Many owners had stated they would like this information, and it provided another opportunity to thank them for taking part.

2.2.9.4 Study documentation and record keeping

Before agreeing to take part in the study, owners were provided with an information sheet (Appendix 1) and details of what would take place over the course of the 12 months (Appendix 2). This information was reiterated at the initial health check appointment prior to the owner signing the study consent form (Appendix 3). Owners were also required to sign the hospital's own consent form prior to the neutering procedure. Their local first opinion veterinary practice was sent documentation to inform them when a dog signed up to the study (Appendix 4), when they were neutered, the results of clinical pathology blood tests and vaccine batch numbers. This was to ensure continuity of care for the dog. The owner signed a consent form if they were happy for this communication to take place (Appendix 5). A questionnaire regarding ETS exposure and dog health was distributed to owners (Appendix 6).

The dogs were each assigned a study number and their information, including demographics and questionnaire responses from their owners, were stored in a Microsoft Access database. The database was only available to view by the author and supervisors. Collaborators were provided only with information required and not allowed database access. However, the owner and pet details were available to staff within the small animal hospital and were stored as a standard clinical record. This was to ensure that staff caring for each dog in the hospital had the information needed to perform their job efficiently. Study documentation completed by the owners was stored in a locked cabinet.

2.2.9.5 The study group and retention rate

42 dogs took part in total. 2 extra dogs (beyond the intended 40) were recruited as it was known that 2 of the dogs who joined the study early on had been relinquished by their owners. There were 5 females and 37 males. Age at the initial health check appointment ranged from 5.5 months to 7 years, with a median age of 1 year 5 months. After the breed criteria changed, many different breeds of dog were recruited. The breeds with more than one dog in the study were: Staffordshire bull terrier (n=12), Staffordshire bull terrier X lurcher (n=2), Border collie (n=4), Yorkshire terrier (n=2), cavalier King Charles spaniel (n=2) and English springer spaniel (n=2). Breeds with one dog in the study were: bearded collie, miniature schnauzer, boxer, cocker spaniel, Patterdale terrier, beagle, Jack Russell terrier, German shorthaired pointer, Rottweiler, Shar-pei, Basset hound, Pomeranian X Border terrier, German shepherd dog X collie, Border terrier X pug, Patterdale terrier X Lakeland terrier, English springer spaniel X German shepherd dog, collie X Labrador retriever and a retriever/Rottweiler X bull mastiff.

Of the 42 dogs, 17 (40.5%) did not return for the 12 month follow up appointment. 11 of the dogs that did not return were Staffordshire bull terriers and one was a Staffordshire bull terrier X lurcher. The other dogs who did not return were a boxer, a Rottweiler, a Yorkshire terrier, an English springer spaniel X German shepherd dog and a Shar-pei. Only 1 of the 5 female dogs returned.

2.2.9.6 Ethics

The project was reviewed by the Home Office veterinary surgeon and the decision was reached to keep the project as part of routine veterinary practice. Ethical approval for the study was obtained from the University of Glasgow School of Veterinary Medicine Ethics Committee.

2.2.10 **Haematology and biochemistry testing of blood samples**

Haematology and biochemistry analyses were performed by the University of Glasgow Veterinary Diagnostics Service. An Advia 120 analyser (Siemens, Erlangen Germany) with veterinary software was used for haematology analysis from whole blood in EDTA. Data were verified by making a blood smear stained with Pappenheim stain, which was assessed by a clinical pathologist.

Concentration of biochemistry measures was measured in serum using a Beckman Coulter AU640 automated analyser (Beckman Coulter, High Wycombe UK).

2.2.11 **Nicotine and cotinine concentration measurement**

Hair samples were sent to the University of Glasgow Forensic Toxicology Service. Samples envelopes were uniquely identified, but no information was provided as to the reported exposure by the dog owners to the laboratory, so the operator would be blind to the history of the dog. The procedure was performed using the methodology by Bawazeer et al. (2012). Briefly, 30mg of hair was weighed into 20ml Chromacol environmental vials. Hair was finely snipped and extraction of nicotine and cotinine performed using 2ml methanol and 1% formic acid. 20µl of supernatant was injected into LC-MS/MS system. Chromatographic separation was performed using a ZIC-HILIC column. Triple quadruple tandem mass spectrometry and Agilent Masshunter software (Agilent, Santa Clara USA) were used for identification and quantitative analyses. The lower limit of quantification for nicotine was 0.01ng/mg and 0.05ng/mg for cotinine. Repeat measures were highly correlated ($R^2 > 0.99$).

In previous studies, hair was initially washed to remove surface nicotine and cotinine, then only nicotine and cotinine in the hair lysate were measured (e.g. Pichini *et al.*, 1997; Apelberg *et al.*, 2012). However, as described in Bawazeer, Watson and Knottenbelt (2012), it is worth including surface hair nicotine and cotinine as dogs may absorb these through self-grooming. Therefore, no initial wash step was included and data were reported as the total hair nicotine and

cotinine concentrations, incorporating surface and hair lysate concentrations together.

2.2.12 **Statistical analysis**

Statistical analyses were conducted using Stata, version 13.1 (StataCorp USA, 2013) and R, version 3.2.2 (R project, 2015). Details of statistical tests used will be given in each corresponding results chapter.

Chapter 3: Quantifying environmental tobacco smoke exposure

3.1 Introduction

3.1.1 Measurement of ETS biomarkers in companion animals

The only biomarkers of ETS measured in published studies of companion animals have been nicotine and its derivatives. These have only been measured in a small number of dog and cat studies. 3 studies were found which examined the concentration of cotinine in urine in companion animals. Roza & Viegas (2007) measured urinary cotinine in 30 Yorkshire terriers, half of which were from homes where residents smoked at least 20 cigarettes per day, half were not exposed to ETS at home. Cotinine was detected in the urine of all dogs in the smoking homes and was not detected in any of the non-ETS exposed dogs. Bertone-Johnson *et al.* (2008) measured cotinine concentration in urine samples from 30 dogs in smoking homes and 33 dogs in non-smoking homes. Owners were asked if anyone in the household smoked in the previous 24 hours, number of cigarettes and if this smoking occurred indoors. Cotinine concentrations were approximately doubled in dogs exposed to any ETS compared to unexposed dogs. Cotinine concentrations were increased in dogs where owners used self-applied lawn care, in brachycephalic dogs compared to mesaticephalic and dolichocephalic dogs and with every category of increasing numbers of cigarettes smoked. Cotinine levels were also negatively associated with increasing dog age. The third study involved the measurement of urinary biomarkers in 61 cats (McNiel *et al.*, 2007). 19 of the cats were from smoking households and the other 42 from non-smoking households. Nicotine, cotinine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) concentrations were measured, and all these were found to be at a higher concentration in urine of exposed cats compared to unexposed cats. While urine measures were successfully used in these studies as a means of determining ETS exposure, the use of urine for this study would not be without issues. Urine in Roza & Viegas (2007) was collected by cystocentesis, which is not without risk, would have ethical considerations and would be likely to be unacceptable to owners. Bertone-Johnson *et al.* (2008) used free catch samples into sterile containers and then immediately stored at -80°C . This would be difficult to facilitate in a series of 15 minute clinic appointments. The final study, McNiel *et al.* (2007) used a combination of free catch and cystocentesis.

An alternative tissue to measure ETS biomarkers in is hair. In a Glasgow study of 38 dogs, hair samples were used to examine the relationship between ETS exposure and hair ETS biomarker concentration (Bawazeer, Watson and Knottenbelt, 2012; Knottenbelt *et al.*, 2012). Nicotine concentration was quantified in samples from all dogs, and nicotine N-oxides, cotinine, nornicotine and nornicotine N-oxide were measured in hair of 7 dogs with smoking owners. Nornicotine was only detected in 2 of the selected 7 dogs. Nornicotine N-oxide was present in 5 of the 7, and in 6 of the 7 cotinine and nicotine N-oxides were present. However, nicotine N-oxides were only present on the surface of the hair and not in a lysate of chopped up hair. Nicotine was present in hair from all ETS exposed dogs. The 38 dogs were divided into 3 groups based on zero exposure, occasional exposure and regular exposure to ETS based on a questionnaire and nicotine concentration measured in all samples. Hair nicotine concentrations were highest in the regularly exposed group. The hair was collected from the neck region, where hair is commonly collected in dogs when blood sampling and was then stored in envelopes at room temperature. This method of collection and storage has advantages over the use of urine, as there is no need for facilities for -80°C storage, the method of collection is quick and simple and the procedure would be acceptable to owners.

3.1.2 Comparison of urine and hair as tissues for ETS exposure within individuals

In addition to the advantages of hair in terms of collection and storage, studies have also compared the ability of hair biomarkers to other tissue biomarkers to reflect ETS exposure level within individuals. When 297 hair samples and 158 urine samples were collected from 322 children aged 3 to 27 months, hair nicotine concentrations better discriminated between exposure levels than urinary cotinine concentrations (Al-Delaimy, Crane and Woodward, 2002). The correlation between hair nicotine concentration and number of cigarettes was stronger than the correlation between urinary cotinine concentration and number of cigarettes. In addition, Nafstad *et al.* (1995) measured hair nicotine concentration in 94 children aged 12-36 months, and cotinine concentration in urine samples from 72 of these children. Urinary cotinine could differentiate

between children exposed to greater than 10 cigarettes per day and zero exposure, but not between zero exposure and between 1 and 10 cigarettes per day exposure, whereas, hair nicotine concentrations could be used to differentiate between the three groups.

Other tissues used to measure ETS biomarkers in humans include blood (Yang *et al.*, 2013), saliva (Curvall *et al.*, 1990), nails (Al-Delaimy *et al.*, 2002), deciduous teeth (Garcia-Algar *et al.*, 2003) and meconium (Mozaner Bordin *et al.*, 2014). In the UK, blood sample collection not specifically related to the dog's health would require a Home Office licence. Studies relating to saliva describe collection methods such as spitting into a tube (Kim *et al.*, 2014), holding a dental roll in the mouth for several minutes (Boyd *et al.*, 1998) and chewing on a cotton roll for one minute (Etter, Vu Duc and Perneger, 2000), all of which would be impractical in a dog. In addition, blood and saliva would reflect short term exposure to ETS. Like hair, toe nails have been used to represent long term exposure to ETS, but the collection of hair would be likely to be less stressful for dogs than nail clipping. Part of the reason for opting for nails over hair in humans is that chemical hair treatments have been shown to have an impact on ETS biomarker levels in hair and the use of nails would avoid this. It is unlikely that the dogs in this study would have had their hair coloured or permed, so this would not be such an issue here.

3.1.3 Measurement of hair ETS biomarkers in humans

Several studies have employed the use of hair samples in humans to assess ETS exposure, some examples of these are presented here. Pichini *et al.* (1997) collected hair samples from 24 infants, aged 3 to 36 months, who were either non-exposed to ETS, occasionally exposed to ETS or regularly exposed to ETS. Nicotine and cotinine concentrations were measured. Nicotine concentrations were significantly different between the 3 different exposure groups, but cotinine was only measured in the regularly exposed infants. Furthermore, in a study by Al-Delaimy *et al.* (2000) hair cotinine was less strongly associated with exposure status than hair nicotine concentration. For this study, hair samples from 112 children, aged 3 months to 10 years, and 76 of their mothers were

collected and measured for nicotine and cotinine concentration. The hair nicotine in children increased with both the number of smokers in the house and the total number of cigarettes smoked at home, and was also strongly correlated to their mother's hair nicotine concentration. Kim et al. (2009) measured hair nicotine concentrations in samples from 1017 children under 11 years of age and 852 non-smoking women living in households with smokers. They also measured air nicotine content in the homes. For every $1\mu\text{g}/\text{m}^3$ increase in air nicotine concentration, there was a 3% increase in hair nicotine concentration in children and only a 1% increase in adult women. In addition, the children who spent over 19 hours a day at home showed increased concentrations of hair nicotine compared to those who did not. This is interesting for our study, as many of the dogs will spend all their time, aside from going on walks, at home.

3.1.4 Aims and hypotheses for this chapter

The aim of this chapter was to establish a reliable biomarker for ETS exposure in the dogs in this thesis. Hair was used as the tissue to measure the biomarker in as it can be used as a long-term representative tissue. Nicotine and its derivatives are the only biomarkers previously measured in companion animals and they have also been widely studied in humans, and therefore nicotine and its metabolite cotinine were measured here.

Owner questionnaire generated data were compared to nicotine and cotinine concentrations in dog hair, to explore whether exposure status reported correlated with nicotine and cotinine concentrations and if there were any further variables outside of smoking habit which were associated with the concentrations. This information was also to be used to create cut-off values for the markers to classify exposure for future studies.

It was hypothesised that hair nicotine concentrations would correlate strongly with increasing ETS exposure, and that cotinine would be increased in those with the highest exposure levels. In terms of other variables, it was hypothesised that smaller dogs would have higher nicotine and cotinine levels, as would those

with shorter noses due to previous findings by Bertone-Johnson *et al.* (2008). It was also hypothesised that those with access to a private garden would have decreased ETS biomarker concentrations, but where owners used lawn chemical products, this would increase cotinine levels. It was also expected that, due to population density, dogs living in urban environments would have higher biomarker concentrations, and dogs living in areas with a lower index of deprivation (indicating lower social mobility) would have increased biomarker levels due to the increased rates of smoking found in these areas on average.

3.2 Methods

3.2.1 Hair nicotine and cotinine

The study dog group was as described in section 2.2.9. Fur samples were clipped from dogs from the neck region prior to blood sampling. Fur was placed in foil and within a sealed envelope to prevent any further smoke exposure. In dogs attending both time points, where possible fur was collected from the same side of the neck. Samples were transferred to the University of Glasgow Medical Forensics department, where nicotine and cotinine concentrations were measured using the method described in section 2.2.11.

3.2.2 Questionnaire and dog demographic details

Dog weight was recorded at both time points. Breed height was estimated for each dog using information from the UK Kennel Club and American Kennel Club websites and individual breed literature (The Patterdale Terrier, no date; Plummer, 1985; American Kennel Club, 2016; The Kennel Club Limited, 2016). For cross breeds, the mean of the parent breed heights was used. Cephalic indices were obtained from Carrasco et al (2014). Mean values were used for cross-breeds. Cephalic index was not available for the Yorkshire terriers, Shar-pei, Staffordshire bull terrier X lurchers, Border terrier X pug, Border terrier X Pomeranian, Patterdale terrier or Patterdale terrier X Lakeland terrier.

Fur colour, type and length were noted at the time of sampling. Lengths were divided into short, medium or long. Shedding rates were obtained from the Purina™ pet food website (Purina, 2016) and divided into low, medium or high. Owner index of deprivation was obtained from the Scottish Neighbourhood Statistics website using their postcodes (The Scottish Government, 2015b).

Questions regarding ETS exposure were as in Knottenbelt et al. (2012) with additional questions relating to time spent with the dog by each smoker, and are shown in Appendix 6, which contains the questionnaire in entirety. ETS

exposure was divided into 3 levels: no exposure at home (group 1), owner or friends/family did smoke but not indoors at home (group 2) and owners smoke inside the home (group 3).

3.2.3 Data analysis

Normality checks were performed on data using a Shapiro-Wilk test. A p value of <0.10 resulted in the variable being treated as non-parametric. The nicotine and cotinine concentrations had a non-parametric distribution, so pairwise comparisons were conducted using a Wilcoxon rank-sum test. Kruskal-Wallis tests were used to compare data in more than 2 groups. If the Kruskal-Wallis test resulted in a p value of less than 0.05, pairwise Wilcoxon rank-sum tests with Holm p value correction were undertaken. To test the correlation between two continuous variables, Spearman's rank correlation was used. In addition, to test which variables were associated with nicotine or cotinine concentrations, generalised linear models were undertaken. Model residuals were checked by using the `plot(modelname)` function in R.

All statistical analyses were performed in R version 3.2.2 (R Core Team, 2014). Statistical significance level was taken as $p < 0.05$.

3.3 Results

3.3.1 Descriptive analysis of cohort

Of the 42 dogs, 22 were reported to have no ETS exposure and were placed in group 1, 6 were exposed to ETS but no smoking occurred inside the house and so were placed in group 2, and 14 were exposed to ETS inside the house and were placed in group 3. Between the two time points, the owners of 4 of the dogs gave up smoking and 3 of these started to use e-cigarettes, placing these 4 dogs in group 1 for the second time point. Consequently, of the 25 dogs which returned for the second time point, 17 were in group 1, 2 were in group 2 and 6 were in group 3. One of the female dogs at time point 1 had insufficient fur sampled for mass spectrometry analysis of nicotine and cotinine concentration. Table 3-1 summarises the demographic variables of the dogs in each group at time point 1 and Table 3-2 summarises the demographics of the dogs attending for the second time point. Table 3-3 details the breeds in each group.

Table 3-1 General description of the dogs attending for the first time point.

Median and interquartile ranges are given, with the p values of Kruskal-Wallis tests between the 3 groups for the variables displayed in the final column under "Difference test"

Time point 1	Overall	Group 1	Group 2	Group 3	Difference test
Number	42	22	6	14	
Males	37	20	4	13	
Females	5	2	2	1	
Nicotine ng/mg	0.14 (0.56)	0.03 (0.03)	0.16 (0.80)	0.73 (1.99)	<0.001
Cotinine ng/mg	0.05 (0.13)	0.00 (0.05)	0.05 (0.24)	0.14 (0.09)	<0.001
Age	1.39 (2.22)	1.76 (2.31)	1.01 (2.31)	1.56 (2.14)	0.62
Weight kg	16.9 (7.2)	14.8 (9.4)	18.9 (5.1)	17.6 (9.0)	0.44
Height cm	39 (18)	39 (16)	54 (29)	38 (19)	0.07
Cephalic index	63.1 (21.7)	56.9 (23.1)	66.6 (22.1)	75.1 (21.3)	0.24
Index of deprivation	2749 (3156)	2912 (2801)	2187 (3076)	662 (2784)	0.16

Table 3-2 General description of the dogs attending for the second time point.

Median and interquartile ranges are given, with the p values of Kruskal-Wallis tests between the 3 groups for the variables displayed in the final column under "Difference test"

Time point 2	Overall	Group 1	Group 2	Group 3	Difference test
Number	25	17	2	6	
Males	24	16	2	6	
Females	1	1	0	0	
Nicotine ng/mg	0.06 (0.31)	0.03 (0.09)	0.52 (0.91)	1.94 (8.31)	0.002
Cotinine ng/mg	0.00 (0.18)	0.00 (0.04)	0.10 (0.20)	0.35 (0.20)	0.01 (with ties)
Age	1.73 (1.78)	1.91 (3.61)	1.55 (0.03)	1.73 (0.18)	0.21
Weight kg	17.8 (11.1)	17.2 (11.1)	26.7 (11.3)	15.5 (10.2)	0.37
Height cm	41 (19)	47 (19)	61 (9)	33 (2)	<0.05
Cephalic index	54.4 (18.1)	53.9 (2.6)	62.6 (14.9)	75.7 (23.8)	0.24
Index of deprivation	2876 (2844)	2880 (2434)	3471 (1698)	770 (3119)	0.63

Table 3-3 Breeds in each exposure group

Breeds are divided by whether they attended both time points or time point 1 only. The 4 dogs in italics changed exposure from group 2 or 3 to group 1 between the time points

Group	Breeds at both time points	Breeds at time point 1 only
1	Pomeranian/Border terrier X (n=1) Bearded collie (n=1) Miniature schnauzer (n=1) Springer spaniel (n=1) Cocker spaniel (n=1) Patterdale terrier (n=1) German shepherd dog/Collie X (n=1) Border terrier/Pug X (n=1) Beagle (n=1) Jack Russell terrier (n=1) German shorthaired pointer (n=1) Shar-pei (n=1) Border collie (n=2) Staffordshire bull terrier (n=1)	Boxer (n=1) Springer spaniel/German shepherd dog X (n=1) Staffordshire bull terrier (n=5)
2	Retriever/Rottweiler & bull mastiff X (n=1) Collie/Labrador retriever X (n=1) <i>Border collie (n=1)</i>	Rottweiler (n=1) Staffordshire bull terrier (n=2)
3	Patterdale terrier/Lakeland terrier X (n=1) Basset hound (n=1) Staffordshire bull terrier/Lurcher X (n=1) Cavalier King Charles spaniel (n=2) Staffordshire bull terrier (n=1) <i>Springer spaniel (n=1)</i> <i>Yorkshire terrier (n=1)</i> <i>Border collie (n=1)</i>	Staffordshire bull terrier/Lurcher X (n=1) Staffordshire bull terrier (n=3) Yorkshire terrier (n=1)

Nicotine values were log transformed for further analyses to improve model residuals. There were no zero values, however there were 15 zero readings for cotinine concentration. The cotinine values were square root transformed to improve model residuals.

Nicotine measurements at the two time points were highly correlated (Spearman's $\rho=0.89$, $p<0.001$, Figure 3.1), as were cotinine measurements at the two time points (Spearman's $\rho=0.85$, $p<0.001$, Figure 3.2). Nicotine concentration and cotinine concentrations were significantly correlated at time point 1 (Spearman's $\rho=0.86$, $p<0.001$, Figure 3.3) and time point 2 (Spearman's $\rho=0.77$, $p<0.001$, Figure 3.4). The hair nicotine concentrations at time point 1 (HNC1) were significantly different between the ETS exposure groups (Kruskal-Wallis test, $p<0.001$, Figure 3.5). Pairwise Wilcoxon rank sum tests with Holm p value adjustment showed there were significant differences in HNC1 between group 1 and 2 ($p=0.02$), and between groups 1 and 3 ($p<0.001$) but no significant difference between groups 2 and 3 ($p=0.22$). There was a significant difference in hair cotinine concentration at time point 1 (HCC1) between the exposure groups as well (Kruskal-Wallis test, $p<0.001$, Figure 3.6). Pairwise Wilcoxon rank sum tests with Holm p value adjustment showed there was only a significant difference in HCC1 between groups 1 and 3 ($p<0.001$), and not groups 1 and 2 ($p=0.53$) or groups 2 and 3 ($p=0.58$). A significant difference in hair nicotine concentration at time point 2 (HNC2) was also observed between the exposure groups (Kruskal-Wallis test, $p=0.002$, Figure 3.7). Pairwise Wilcoxon rank sum tests with Holm p value adjustment showed a significant difference between group 1 and 3 ($p=0.004$), but not between group 2 and 3 ($p=0.29$) or group 1 and 2 ($p=0.29$). In addition, there was a significant difference in hair cotinine concentration at time point 2 (HCC2) between the exposure groups ($p=0.01$, Figure 3.8). Likewise, as at time point 1, pairwise Wilcoxon-rank sum tests showed a significant difference in HCC2 between group 1 and 3 only ($p=0.02$), and not between groups 1 and 2 ($p=0.48$) or groups 2 and 3 ($p=0.48$). While neither HNC or HCC at either time point could discriminate between groups 2 and 3, it should be noted that there were a relatively small number of dogs in group 2 (6 at time point 1, 2 at time point 2), which will have impacted the analyses.

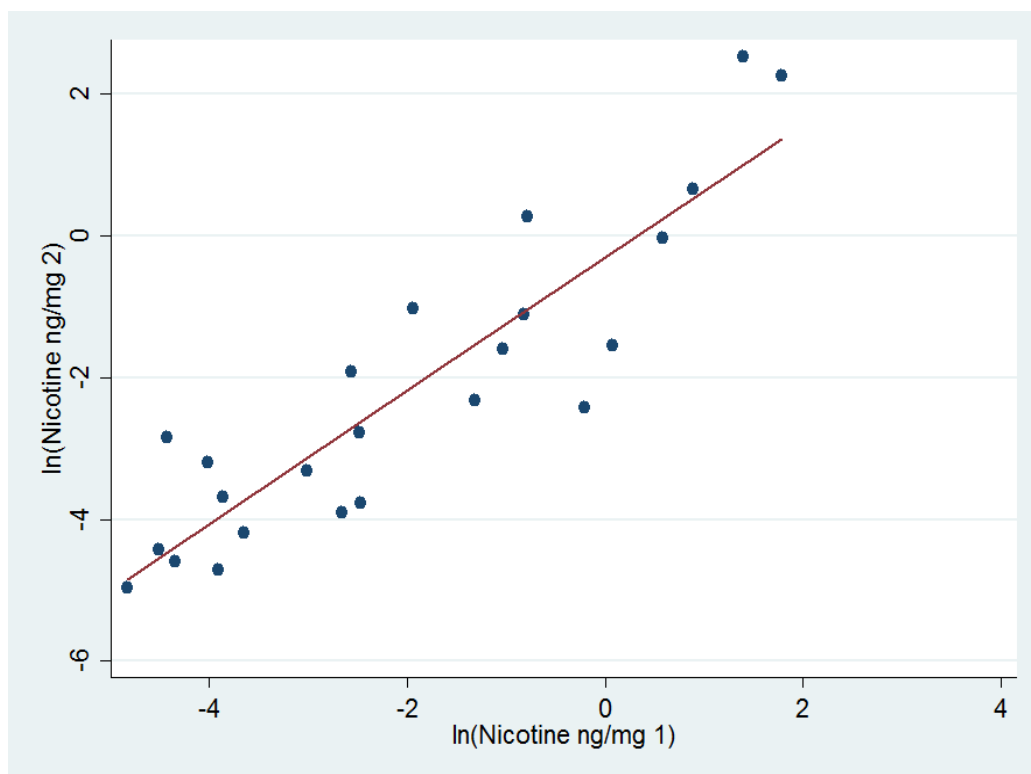


Figure 3.1 HNC at time points 1 and 2 within each dog

Paired HNCs including dogs which changed exposure groups during the study. Measurements were highly correlated (Spearman's $\rho=0.89$, $p<0.001$). $N=25$ (24 male, 1 female).

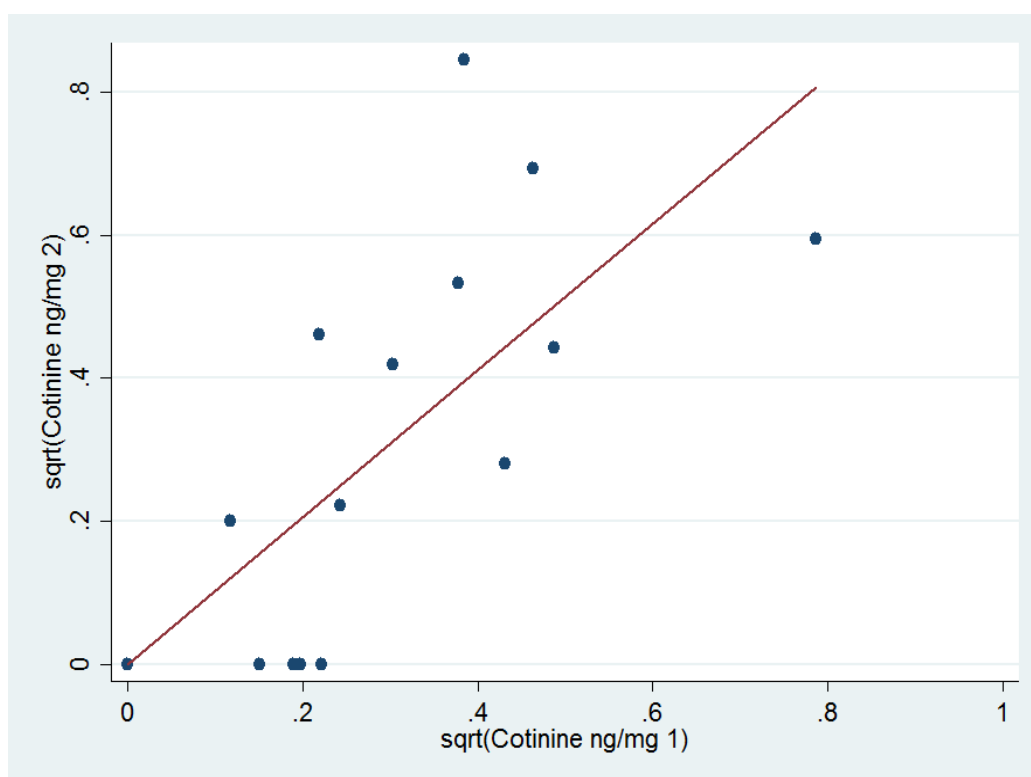


Figure 3.2 HCC at time points 1 and 2 within each dog

Paired HCCs including dogs which changed exposure groups during the study. 11 dogs had HCC of 0ng/mg at both time points. The correlation was statistically significant (Spearman's $\rho=0.85$, $p<0.001$). $N=25$ (24 male, 1 female).

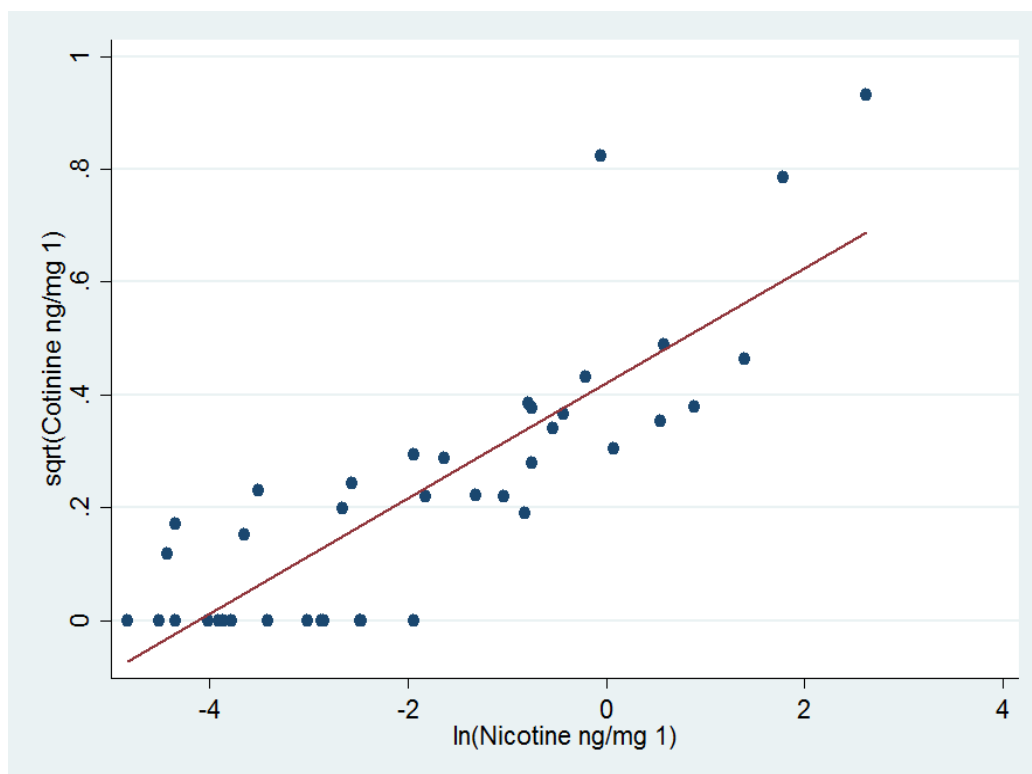


Figure 3.3 HNC and HCC measurements at time point 1

The two measures were significantly correlated (Spearman's $\rho=0.86$, $p<0.001$). N=41 (37 male, 4 female).

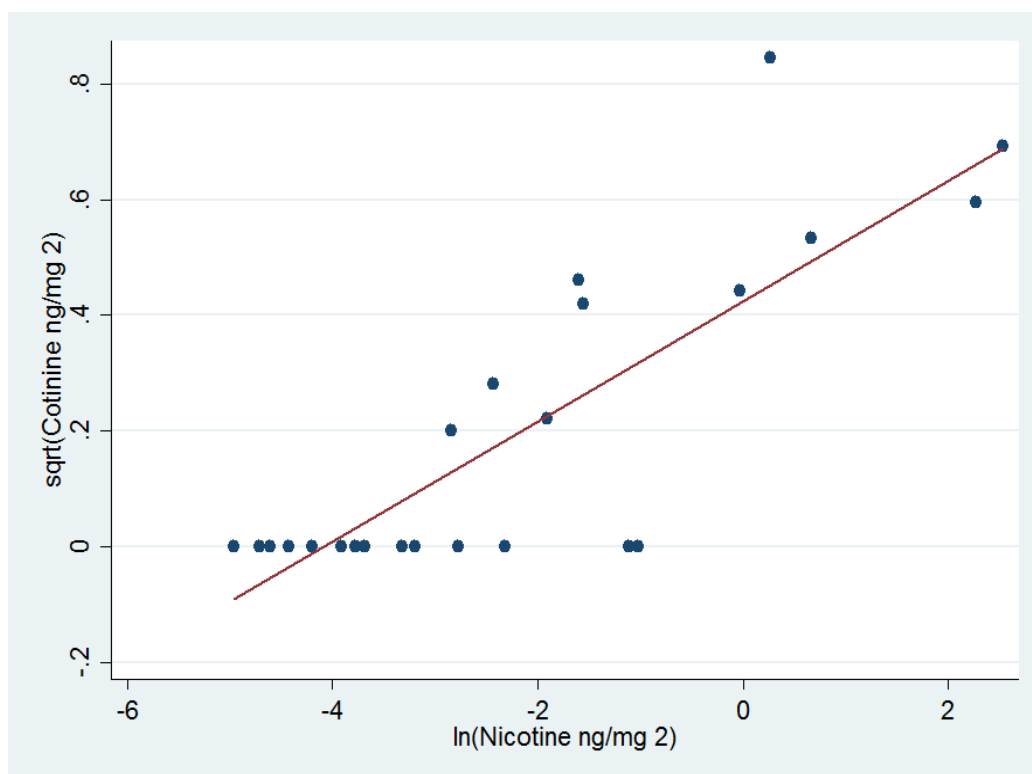


Figure 3.4 HNC and HCC measurements at time point 2

The two measures were significantly correlated (Spearman's $\rho=0.77$, $p<0.001$). N=25 (24 male, 1 female)

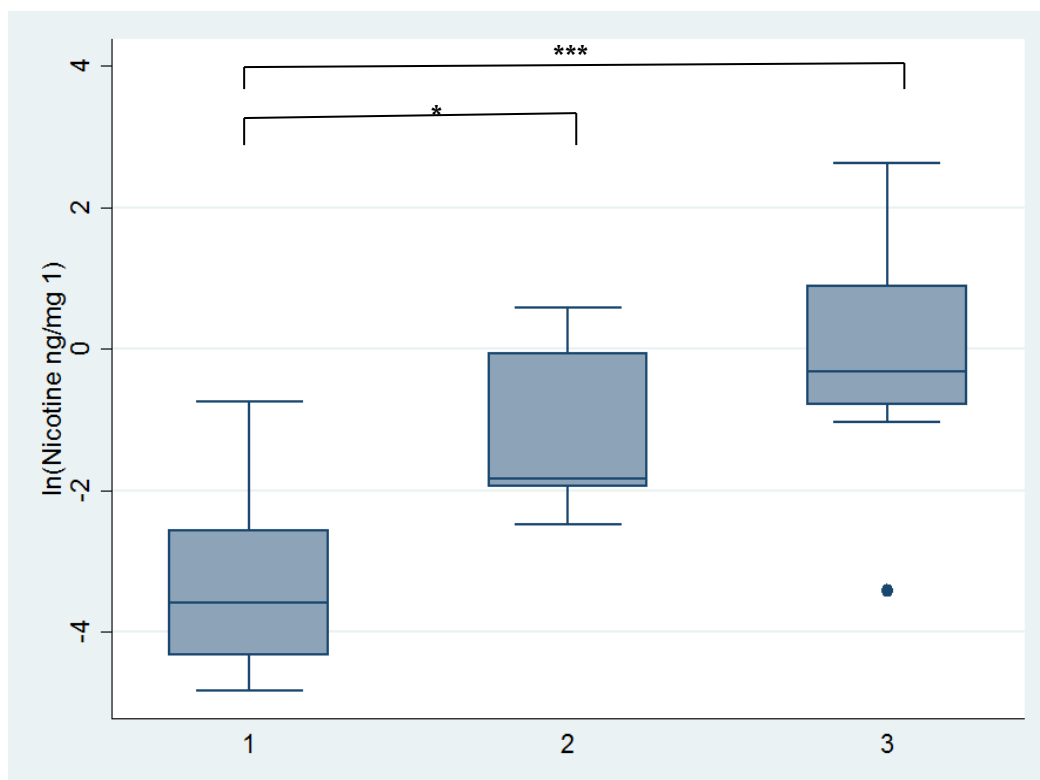


Figure 3.5 HNC at time point 1 by exposure group

Pairwise differences are indicated by *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. N=41 (37 male, 4 female)

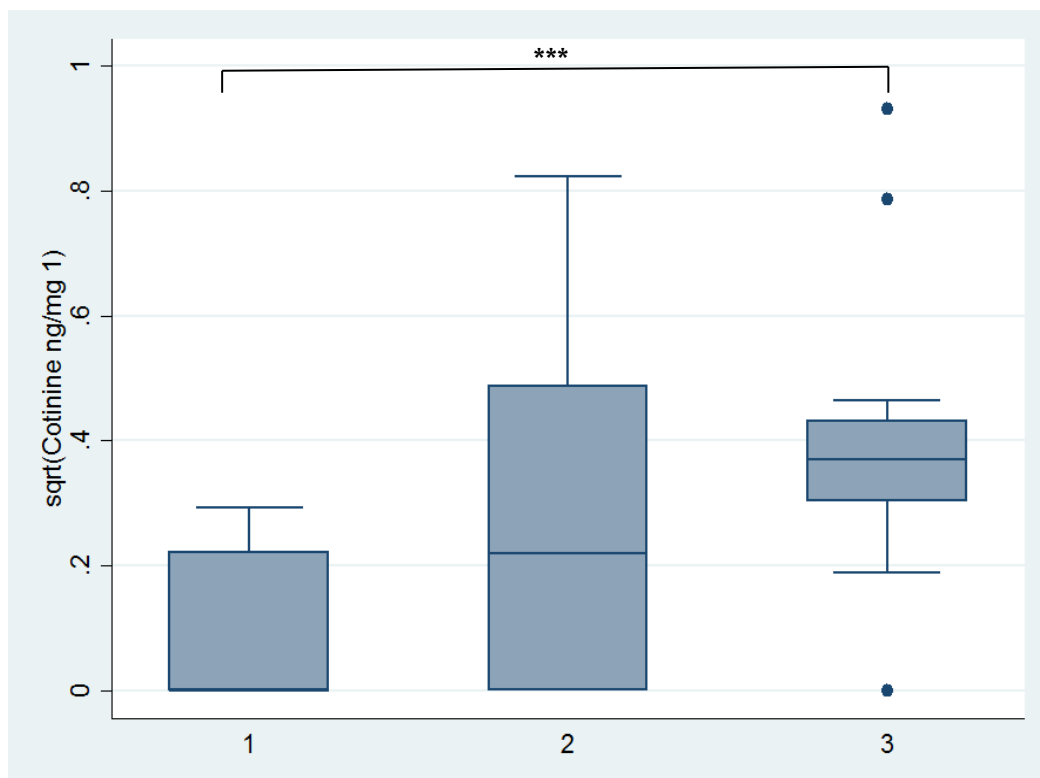


Figure 3.6 HCC at time point 1 by exposure group

Pairwise differences are indicated by *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. N=41 (37 male, 4 female)

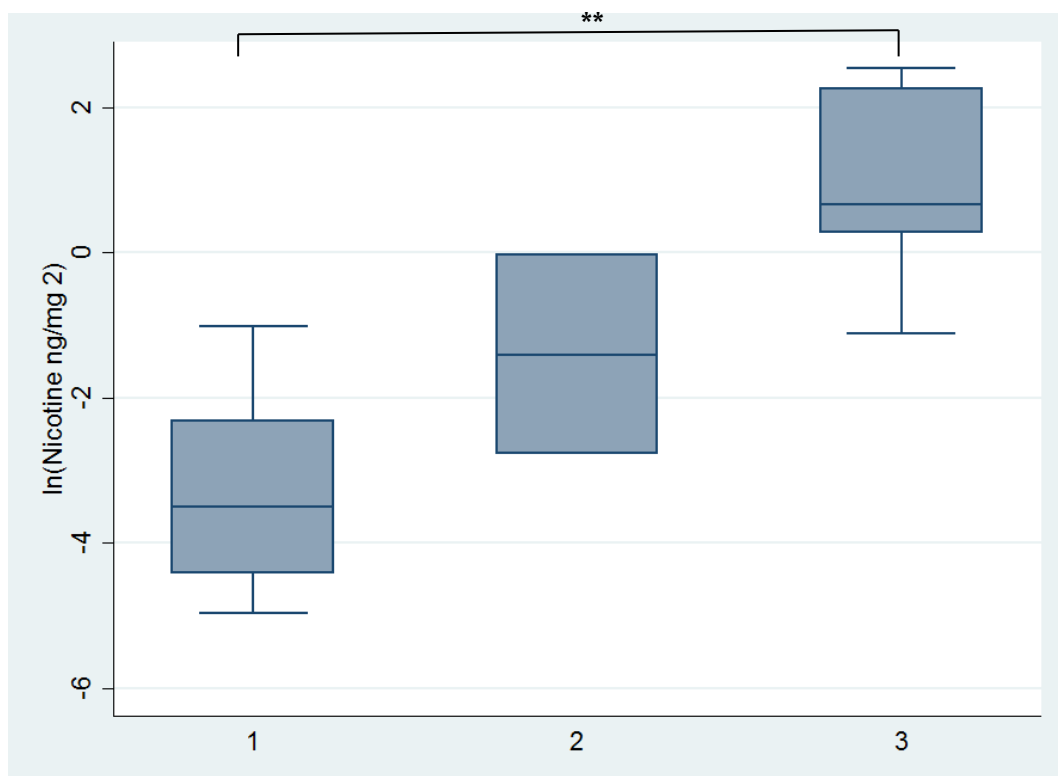


Figure 3.7 HNC by exposure group at time point 2

Pairwise differences are indicated by *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. N=25 (24 male, 1 female)

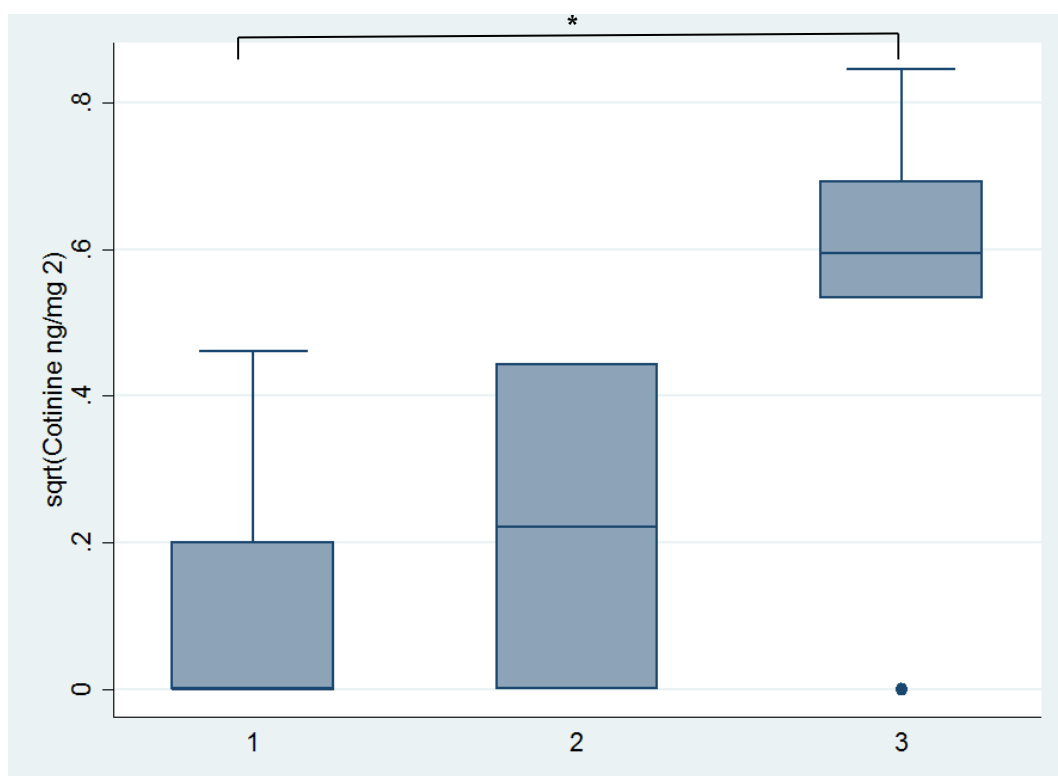


Figure 3.8 HCC by exposure group at time point 2

Pairwise differences are indicated by *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. N=25 (24 male, 1 female)

3.3.2 ROC analysis

To use HNC and HCC as biomarkers of ETS exposure, without owner history, ROC analysis was performed to determine positive and negative cut off values which would result in the highest percentage of dogs being correctly classified as being exposed or unexposed. For this section, groups 2 and 3 were combined in one “smoking home” group. Figures 3.9-3.12 display the generated ROC curves. Using a cut-off value of 0.357ng/mg at HNC1 resulted in 87.80% of dog being correctly classified as being in smoking or non-smoking homes. Cut off points of 0.328ng/mg and 0.974ng/mg for HNC2 resulted in 92.0% of the dogs being correctly classified. Using a cut off of 0.328ng/mg for HNC at both time points did not alter the percentage of dogs correctly classified at time point 1. For HCC1, the optimum cut off was 0.092ng/mg, which correctly classified 85.37% of the dogs. The cut off was higher for HCC2, with a cut off of 0.196ng/mg or 0.284ng/mg correctly classifying 88.0% of dogs. If the same cut off as HCC1 was used, 0.092ng/mg, 80.0% of dogs would be correctly classified. If the 0.196ng/mg cut-off point generated from time point 2 data was applied to the time point 1 data, only 68.3% of dogs would be correctly classified, and the sensitivity would be just 0.32. Those in homes where the owners had either stopped smoking or switched to using e-cigarettes between the two time points were placed in the non-smoking home group for the time point 2 analyses. If these dogs were placed in the smoking group at time point 2, a cut off of 0.079ng/mg resulted in the correct classification of 88.0% of dogs for HCC2.

Tables 3-4 to 3-7 tabulate the exposed and unexposed by if they were test positive or negative for HNC1, HNC2, HCC1 and HCC2. These figures were used to calculate sensitivities and specificities, with 95% confidence intervals, which are shown in Table 3-8 and Table 3-9.

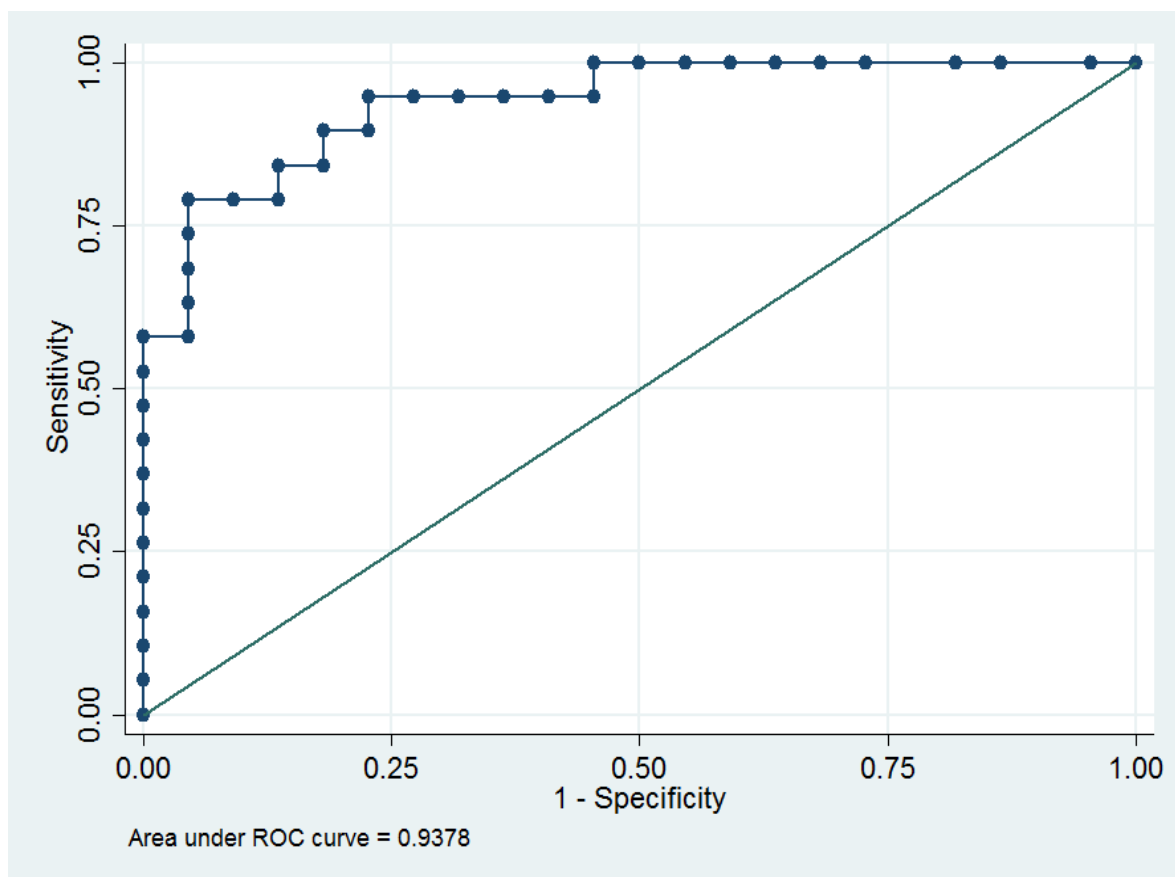


Figure 3.9 ROC curve of HNC1

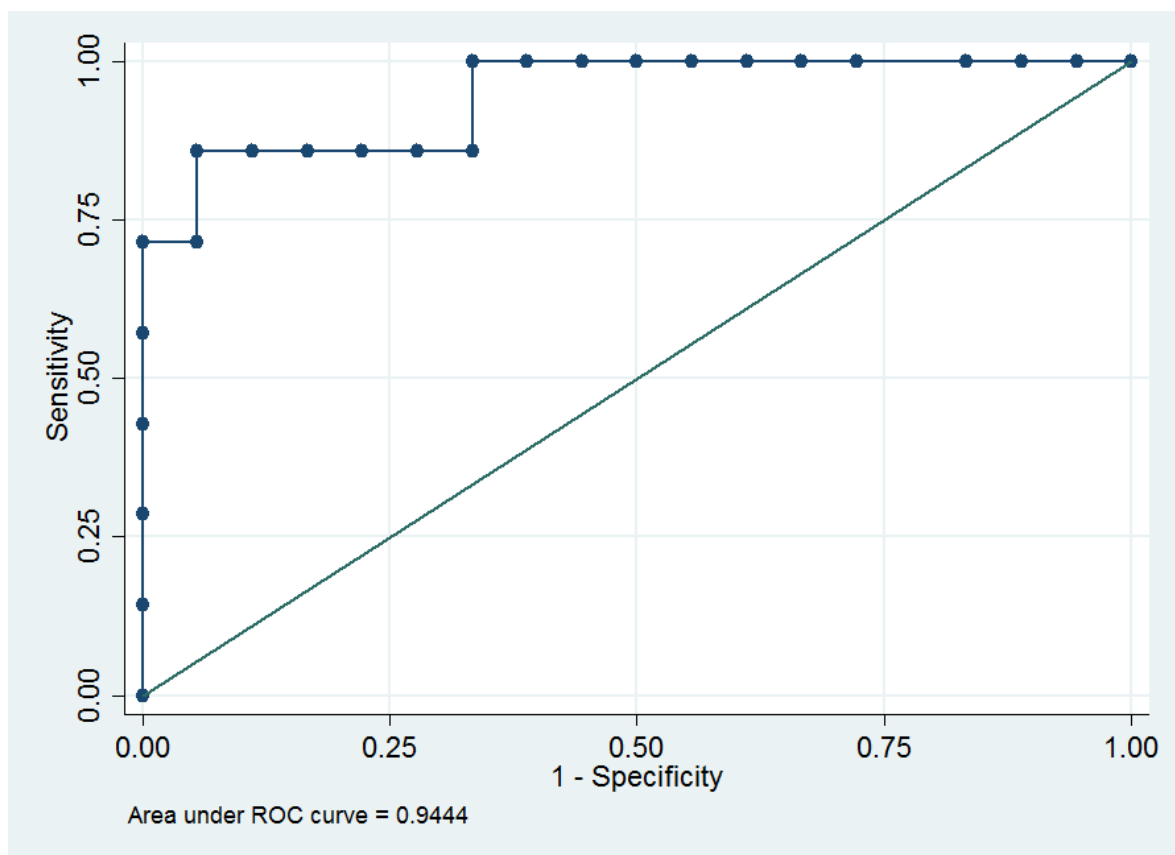


Figure 3.10 ROC curve of HNC2

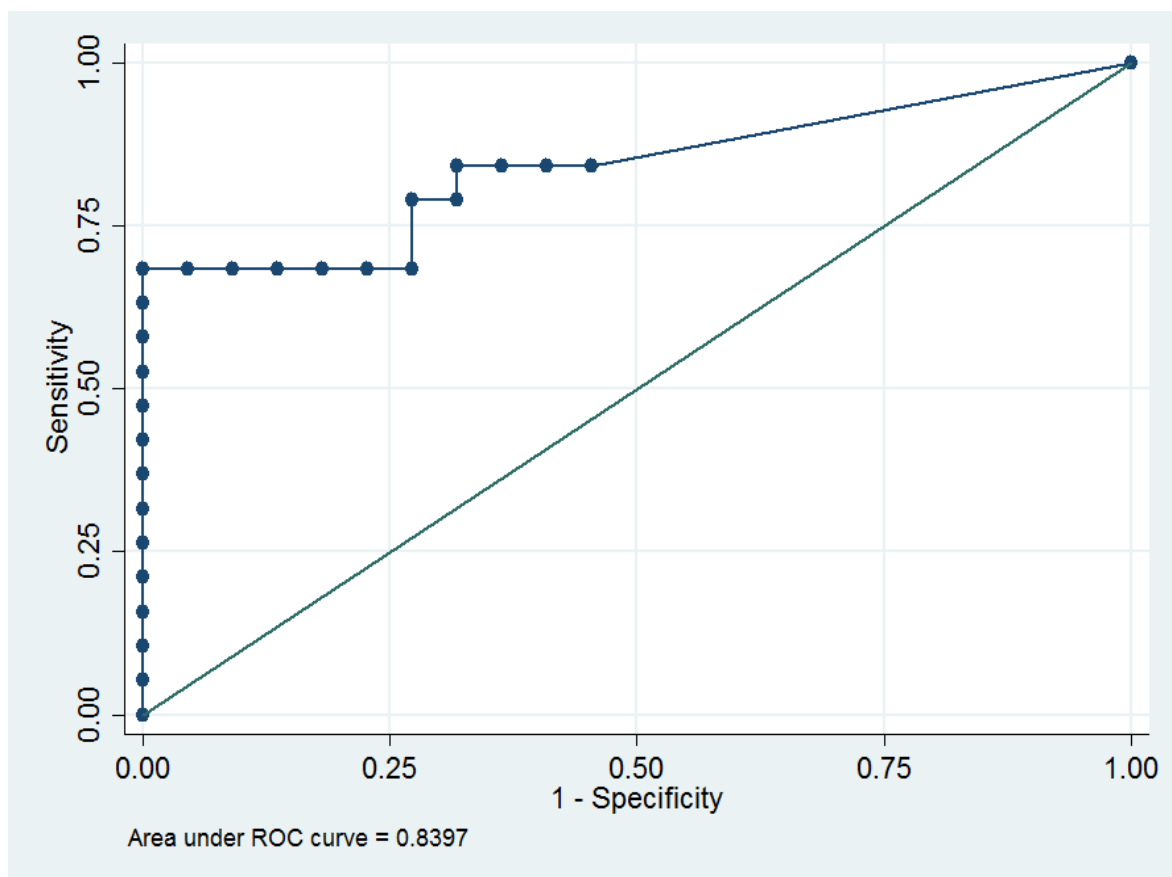


Figure 3.11 ROC curve of HCC1

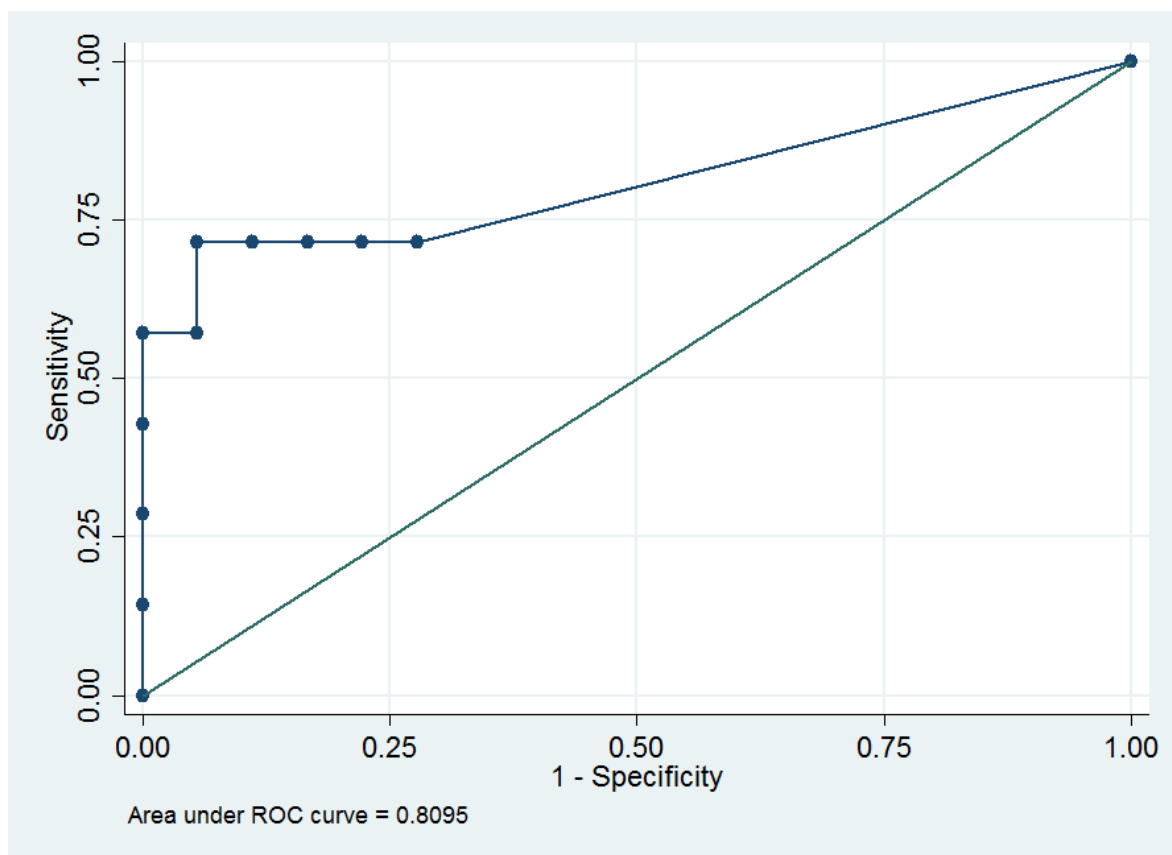


Figure 3.12 ROC curve of HCC2

Table 3-4 Nicotine concentration at time point 1: positive and negative by exposure

Cut off 0.328ng/mg	Smoking home	Non-smoking home	Totals
HNC1 positive	15	1	16
HNC1 negative	4	21	25
Totals	19	22	41

Table 3-5 Nicotine concentration at time point 2: positive and negative by exposure

Cut off 0.328ng/mg	Smoking home	Non-smoking home	Totals
HNC2 positive	6	1	7
HNC2 negative	1	17	18
Totals	7	18	25

Table 3-6 Cotinine concentration at time point 1: positive and negative by exposure

Cut off 0.092ng/mg	Smoking home	Non-smoking home	Totals
HCC1 positive	13	0	13
HCC1 negative	6	22	28
Totals	19	22	41

Table 3-7 Cotinine concentration at time point 2: positive and negative by exposure

Cut off 0.196ng/mg	Smoking home	Non-smoking home	Totals
HCC2 positive	5	1	6
HCC2 negative	2	17	19
Totals	7	18	25

Table 3-8 HNC1 and HNC2 test properties

HNC1 cut-off 0.328ng/mg	Value	95% confidence interval
Sensitivity	0.79	0.54 - 0.93
Specificity	0.95	0.75 - 1.00
HNC2 cut-off 0.328ng/mg	Value	95% confidence interval
Sensitivity	0.86	0.42 - 0.99
Specificity	0.94	0.71 - 1.00

Table 3-9 HCC1 and HCC2 test properties

Described using the different cut off points generated by ROC analysis

HCC1 cut-off 0.092ng/mg	Value	95% confidence interval
Sensitivity	0.68	0.43 - 0.86
Specificity	1.00	0.82 - 1.00
HCC2 cut-off 0.196ng/mg	Value	95% confidence interval
Sensitivity	0.71	0.30 - 0.95
Specificity	0.94	0.71 - 1.00
HCC2 cut-off 0.092ng/mg	Value	95% confidence interval
Sensitivity	0.71	0.30 - 0.95
Specificity	0.89	0.65 - 0.98

3.3.3 Factors related to HNC and HCC

No dog had a zero value for HNC at either time point, regardless of exposure. The previous study conducted in Glasgow on hair nicotine concentrations found zero values for HNC (Knottenbelt *et al.*, 2012). Therefore, in the dogs that were not exposed to ETS at home, the factors which were associated with their HNC and HCC measures were investigated. In addition, analyses were conducted to see if there were variables which were related in HNC and HCC in ETS exposed dogs. The description of these analysis is given in Appendix 7.

3.3.4 Change in exposure status

4 of the dogs had a change in exposure between the two time points, with 3 of the owners switching from conventional cigarettes to e-cigarettes. All 3 of the dogs where owners switched to e-cigarettes showed a decrease in HNC between the two time points, such that at time point 1 they were in the positive HNC group, and at time point 2 they were in the negative HNC group (using ROC generated cut-off points). However, only one of these dogs demonstrated a decrease in HCC, and the other two showed an increase. One of the dogs had a four-fold increase in HCC between the two time points. E-cigarettes do still contain nicotine, and therefore could result in cotinine in the dogs' hair, but given that the owners were using e-cigarettes as a means to stop smoking it seems unlikely that cotinine levels would increase from the e-cigarette exposure alone. With such small numbers, it is difficult to comment on the reasons behind the cotinine concentrations in these dogs. The dog where the owner stopped smoking during the year had an increase in HNC between the two time points. This owner was unsure of ETS exposure their dog was experiencing from smoking habits of their partner and visitors, so the increase in HNC during this time may be due to exposure from people other than the owner smoking. This dog had an HCC of 0ng/mg at both time points.

3.4 Discussion

3.4.1 Questionnaire design

The questionnaire covered many areas. However, for future studies, an editing of the questionnaire would be helpful to reduce the time owners need to engage in questionnaire responding and keep their interest. Little was gained from knowing smoking location habits other than to know if owners acted to protect their family from smoking in some way, such as only smoking outdoors. Several owners did not own cars, for example, so this question was not helpful and embarrassed some. In others, behaviours such as smoking in a car nor in close contact with their dog was in keeping with smoking indoors. Given the size of the group and numbers who smoked, further division due to these types of behaviours did not aid analysis.

The number of cigarettes groups for each smoker were too broad. Most smoking owners smoked 20 cigarettes or less per day. Given that there was a relationship between the number of cigarettes smoked in the household per day and nicotine and cotinine measures, it may have been useful to know in more detail the number of cigarettes smoked, rather than using wide groups. However, it was difficult to get some owners to quantify their habits, due to perhaps embarrassment or variability day-to-day, so using wide groups helped to glean information from owners without them feeling judged. In addition, those who smoked roll-your-own cigarettes found it difficult to quantify their habits, and more than one of these smokers felt that their type of cigarettes “did not count” in terms of environmental tobacco smoke exposure, and that passive smoking was only an issue in factory-made cigarettes. In hindsight, it would have been helpful to research common quantities of tobacco used by roll-your-own cigarette smokers and to have a box for recording quantities of tobacco pouches used per week. Data from the International Tobacco Control Europe Survey showed that between 2007 and 2010, exclusive roll-your-own cigarette use among smokers increased from 26.4% to 32.7% (Brown *et al.*, 2015). In addition, the Office for National Statistics Opinions and Lifestyle Survey found that in 2014, 39% of male smokers and 28% of female smokers used roll-your-own cigarettes in Great Britain (Office for National Statistics, 2016). No data could

be found for the rate of roll-your-own cigarette use in Scotland alone, but these figures suggest that future studies should be designed with roll-your-own cigarette users in mind. Tobacco used per cigarette would vary between roll-your-own smokers (Darrall and Figgins, 1998), so asking about weight of tobacco used per week may be a more practical approach than cigarette numbers.

This study is not alone in finding roll-your-own smokers believing them to be less harmful. In a survey of 13,322 adults from Australia, Canada, the United Kingdom and the United States, participants were asked which out of factory-made cigarettes, roll-your-own cigarettes, pipes and cigars they thought were the least and the most harmful. Smokers from the UK were the most likely to report roll-your-own cigarette use of the four countries. White male smokers under 25 from the UK or Australia who smoked roll-your-own cigarettes sometimes or exclusively were the most likely to see roll-your-own cigarettes as the least harmful (O'Connor *et al.*, 2007).

3.4.2 Dogs of owners giving up smoking

Three of the dogs went from heavy smoke exposure to e-cigarette exposure approximately half way between the two time points. The nicotine concentration of these dogs reflected these changes. However, the cotinine concentrations did not, with two of the three dogs having an increase in cotinine concentration. This may be in part due to the longer half-life of cotinine and no sources of information regarding the half-life of nicotine and cotinine in hair following habitual exposure. Alternatively, the cotinine could be due to lingering third-hand smoke in the environment. Nicotine has been measured in the dust of homes both in homes where smoking occurs indoors and homes where adults attempted to protect their families from smoke exposure (Matt *et al.*, 2004) and third-hand smoke was demonstrated in homes where smokers had moved out 2 months prior to measurement (Matt *et al.*, 2011). The owners switched to e-cigarettes as part of their cessation. The cotinine could be due to exposure to the e-cigarette, as serum cotinine levels were similar when passive smoking occurred from a tobacco cigarette and an e-cigarette in humans (Flouris *et al.*, 2013). However, salivary cotinine levels were found to be significantly lower in non-smokers exposed to e-cigarettes compared to tobacco cigarettes at home in another study (Ballbè *et al.*, 2014) and e-cigarette emissions have been

shown to be far safer than tobacco cigarettes in terms of risk to human health (McAuley *et al.*, 2012). Regardless, the demonstration of reduction in hair nicotine of the dogs could be motivation for the owners to retain their cessation habit, and future projects which employ feeding back to owners the levels of their dog's hair nicotine as a means of helping them stop smoking could be worth pursuing.

The owner of one dog changed smoking habit from smoking only outside to not smoking. The HNC of the dog actually increased between the two time points. When speaking to the owner at the second time point, they were unsure of the ETS exposure of the dog from their partner and visitors to the house when they were not present. By using HNC as the measure of ETS exposure rather than owner reported exposure, uncertainties such as these will not impact analyses in studies evaluating relationships between ETS exposure and biological measures in the dog.

3.4.3 Nicotine and cotinine hair concentrations as biomarkers of ETS

Nicotine and cotinine concentrations reflected the ETS exposure, increasing with number of cigarettes smoked in the home. As predicted, nicotine was a better reflection of exposure, and cotinine concentration increased in higher exposures. However, unlike Knottenbelt *et al.* (2012) there were no zero readings for hair nicotine. This may be in part to the measurements being reported as a combination of wash and lysate measurements rather than separately. If they had been reported separately, this may have given further information on why the non-ETS exposed dogs did not have zero nicotine readings.

There were 3 dogs which had negative cotinine concentrations according to the ROC generated cut-off, but were exposed to ETS within the home. A possible reason for this is these dogs having a variation in their cytochrome P450 2A6 enzyme, which catalyses the main route of nicotine metabolism to cotinine.

Variations in this enzyme have been demonstrated in humans (Tricker, 2003). Without further experiments, it is not possible to know if this is the case.

An interesting finding during the study was that weight was positively associated with nicotine concentration in non-ETS exposed dogs, whereas it was negatively related with nicotine in ETS-exposed dogs. Smaller dogs in smoking homes may have increased exposure if they are close to the floor where there may be increased third-hand smoke exposure, and they may have more lap-dog type behaviours, making them physically closer to smokers. Whereas, in non-smoking homes, larger dogs may spend more time exercising outside the home, potentially interacting with smokers or being exposed to sources of ETS exposure.

The ratio of nicotine to cotinine was lower in female dogs, which could reflect an increased activity of cytochrome P450 2A6, with increased conversion of nicotine to cotinine. This has been observed in human females as well (Benowitz *et al.*, 2006), with the clearance of nicotine to cotinine being significantly higher in women than men.

No significant differences were found due to coat colour or other coat variables. This is likely to have been due to the wide variety of coats seen in the study dogs, such that the numbers in each group were very small. Seven genes which determine coat colours and patterns in dogs have been identified, so a great variety is possible (Schmutz and Berryere, 2007). Significant differences in nicotine and cotinine in hair of nicotine exposed pigmented and albino rats were noted in a previous study (Gerstenberg *et al.*, 1995) and nicotine and cotinine adducts have been demonstrated in a melanin intermediate (Dehn *et al.*, 2001), suggesting melanin content of the coat may have an impact on nicotine and cotinine concentration. Examining different colours of hair within the same dog may indicate whether hair colour is confounding the results.

3.4.4 Chapter summary

This chapter examined the use of hair nicotine and cotinine as biomarkers of ETS exposure in pet dogs. Hair nicotine concentration demonstrated the best reflection of ETS exposure in smoke exposed dogs, but positive readings were found in non-ETS exposed dogs. Cotinine was not present in the hair of many non-ETS exposed dogs, but also in some of the dogs with reported ETS exposure. Cut-off points for nicotine and cotinine hair concentration for ETS exposure status were generated using ROC analysis. Over 85% of dogs were correctly classified using a nicotine concentration cut off. If owners changed smoking habits between the two time points, nicotine better reflected the changes than cotinine concentration.

By having biomarkers measured by an outside laboratory, this provided an objective reading of ETS exposure to be used for analysis in the subsequent chapters.

Chapter 4: Optimisation of telomere length measurement method

4.1 Introduction

4.1.1 Methods of telomere length measurement

4.1.1.1 Terminal restriction fragment (TRF) analysis/Southern blot

TRF analysis was the first method used to measure telomere length and was successfully used as a means of demonstrating the shortening of telomeres in human fibroblasts with serial passaging (Harley, Futcher and Greider, 1990). As the first available method for telomere length analysis, it is often used as a reference to which to compare new methods and it is regarded as the “gold standard” method of telomere measurement (Aviv, 2009). Because of this, TRF analysis was selected from the two methods cited in the literature for measuring canine telomere length and optimised for use in this project. However, it could not be the sole method used as the procedure requires substantial amount of DNA and the quantities required were not always successfully extracted from clinical samples. The minimum amount of DNA needed for the method in our laboratory was 1µg, at a minimum concentration of 58.82ng/µl (i.e. 1µg in 17µl). Several of the DNA extractions performed on blood and buccal samples fell short of this target and, given the nature of clinical sample collection, it is not possible to simply collect more.

4.1.1.2 STELA

Baird et al (2003) developed the single telomere length analysis (STELA) method. A telomere homologous 7 based linker is annealed to the 3' overhang of the telomere, followed by a 20 nucleotide non-complementary tail. The linker is then ligated to the 5' end of the chromosome, resulting in the telomere being effectively tagged with a non-complementary tail. PCR is then performed, with a primer of the matching sequence to the tail and a primer which is chromosome specific and located in the subtelomeric region. The products are resolved by agarose gel electrophoresis, Southern blotted and probed with the particular subtelomeric sequence. The resulting bands each represent a single telomere from an individual chromosome. The subtelomeric region needs to be well

characterised and unique to the selected chromosome, which may cause issues when applying the technique in some organisms without a well characterised genome (Baird *et al.*, 2003; Nussey *et al.*, 2014).

Very small amounts of DNA are required for STELA and the method is good at identifying short telomeres, unlike traditional Southern blotting which can be biased against short telomeres (Bendix *et al.*, 2010). Short telomeres are important in cell viability and chromosome stability (Hemann *et al.*, 2001). A method adaptation published in 2010, “Universal STELA”, demonstrates the presence of critically short telomeres regardless of which chromosome they are on, as opposed to the original method which is chromosome specific (Bendix *et al.*, 2010). Telomeres longer than 20kb, however, are not readily identified using STELA which could impact upon analysis of telomeres in small mammals with long telomeres (Aubert, Hills and Lansdorp, 2012).

STELA is technically difficult to optimise (Aubert, Hills and Lansdorp, 2012) and it was felt insufficient technical support would be available to implement this method. In addition, there are no examples of the use of STELA on canine DNA in current literature and canine telomeres have been measured as longer than 20kb in previous studies (e.g. Cadile *et al.* 2007).

4.1.1.3 qPCR

Cawthon (2002) developed a simple high throughput quantitative PCR (qPCR) method for measuring the factor by which telomere length differs between a sample of interest and a reference sample. Telomere and single gene copy primers are used and the relative copies of each compared to the reference sample are taken into account to create a relative telomere to single copy gene (T/S) ratio or “relative telomere length” (RTL). The ratio is proportional to the average telomere length in the sample. When the RTL is 1, the telomere repeat to single gene copy number is the same in the sample of interest and the reference sample.

The numerical differences in RTL between individuals corresponds to the relative difference in their telomere lengths.

qPCR for telomere length is widely used in epidemiological studies due to the relatively low cost, requirements for small amounts of DNA, relative ease compared to techniques like q-FISH and short time to run (Nussey *et al.*, 2014). In addition, it has been used with samples from a variety of organisms including chimpanzees (Tackney *et al.*, 2014), badgers (Beirne *et al.*, 2014), humpback whales (Olsen *et al.*, 2014), warblers (Barrett *et al.*, 2013) and, importantly for this study, dogs (Fick *et al.*, 2012). For these reasons, the method was optimised for use in this study.

One of the main drawbacks of qPCR is the variability between samples and assays (Aubert, Hills and Lansdorp, 2012). In addition, using the Cawthon method the reported value is relative to a reference sample of the laboratory's choosing, meaning ratios cannot be compared between laboratories. In an attempt to overcome this, standard curves of oligo standards to measure “absolute telomere length” have been used (O’Callaghan *et al.* 2008) and this method was used by Fick *et al* (2012) to measure LTL in dogs of a variety of breeds.

4.1.1.4 Q-FISH and flow FISH

Quantitative fluorescent *in situ* hybridisation (Q-FISH) is a method of studying telomere repeats at individual chromosomes, developed by Lansdorp *et al* (1996). The technique uses labelled (CCCTAA)₃ peptide nucleic probes that specifically hybridise to denatured telomere DNA repeat arrays. Fluorescence intensity of bound probe is used to measure telomere length. Samples can be either cultured cells arrested in metaphase or fixed tissue sections.

The method was adapted to measure the average telomere length of cells, by combining the fluorescent in situ hybridisation procedure with flow cytometry. This technique is known as flow FISH (Rufer *et al.*, 1998). The fluorescence level is measured by flow cytometry and in addition different cell populations within a sample can be sorted. Blood samples can be used with this adapted method and age-dependent LTL has been demonstrated with this procedure using human (e.g. Canela *et al.* 2007) and baboon (Baerlocher *et al.*, 2003) samples.

The main drawback with using flow FISH for the canine blood samples in this study is that samples need to be processed within 24 hours of collection (Baerlocher *et al.*, 2006). As surplus blood samples from the University of Glasgow Veterinary Diagnostics Service (VDS) were to be used for technique optimisation, which are only available a week after collection, this would not be possible to achieve. In addition, Baerlocher *et al* (2006) state that “a few ml of blood” would be sufficient for the technique, but the volume of blood spare after clinical pathology testing in the dog is invariably a fraction of this amount.

4.1.1.5 Sequencing

There are a small number of studies in the literature where whole genome sequencing was utilised to determine telomere length. Castle *et al* (2010) counted and normalised the number of sequence reads containing (TTAGGG)₄ in cell line and human DNA. Parker *et al* (2012) improved the method by also including the complementary sequence (CCCTAA)₄ in their counts. They applied their method to paediatric cancer sequences and normal DNA from adults, and were able to demonstrate age-dependent telomere length changes. Improvements in software have taken place so that pure telomere length can be better distinguished from interstitial telomere repeats (Nersisyan and Arakelyan, 2015). The main reason that this method was not be employed in this thesis is the high financial cost associated with whole genome sequencing, which exceeded the study budget.

4.1.1.6 Methods not widely used in literature

The following methods are those which have been described in publications, but then not regularly applied in subsequent studies. For this reason, these methods will not be used in this study.

Dot blot

The dot blot method was designed by Kimura and Aviv (2011) with the aim of developing a procedure which was easy to perform, inexpensive and requiring a relatively small amount of DNA. Analysis is carried out using dot blots. DNA content in each dot is measured by a DNA stain and telomere probe. The telomere length is then normalised to the DNA content. On investigation, the DNA stain used by the authors was not currently available from the listed supplier.

Hybridisation protection assay

In the hybridisation protection assay (HPA), an acridinium ester labelled telomere probe is hybridised to the sample. Chemiluminescence is then measured. An Alu probe is also used to normalise the telomere measurement to the amount of DNA in the sample. Purified, sheared DNA can be used or unpurified DNA in cell or tissue lysate. The process is simple and rapid, and measurements have been shown to correlate well with those using Southern blot TRF analysis (Nakamura *et al.*, 1999).

Enzyme hybridisation assay

The enzyme hybridisation assay for telomere measurement was developed by Freulet-Marriere *et al* (2004). A 96 well plate is coated with a captor phosphodiester oligonucleotide. A telomere tracer oligonucleotide is hybridised to cell lysate samples and a streptavidin-acetylcholinesterase conjugate is used for colorimetric detection. Optical density (OD) measure represents the number of

telomere units in the cell lysate sample and is normalised to the sample DNA concentration.

Primed *in situ*

Cycling-primed *in situ* (PRINS) involves the use of synthetic oligonucleotide telomere primers, which are annealed to sample chromosomes and elongated by the addition of fluorescence labelled nucleotides. The fluorescence signals are detected by fluorescence microscopy. The original method was developed in a Chinese hamster tumour-derived cell line (Musio and Rainaldi, 1997). When human cells (which have much shorter telomeres than hamsters) were used, the primer annealed unevenly. The method was adapted to include primers with both telomere forward and complementary sequences which improved labelling efficiency of human telomeres (Yan *et al.*, 2004).

Detection of individual telomere lengths

This method is based on the Southern blotting protocol, with modification in the sample preparation. A chemistry-based DNA cutter is used to separate the telomere from the rest of the chromosome producing a “real telomere fragment”. The reaction mixture is then treated as per the TRF analysis procedure, run out on a gel and detected by Southern blotting. This method avoids the inclusion of the subtelomeric region as occurs in traditional Southern blotting (Ishizuka, Xu and Komiyama, 2013).

Using surfaced enhanced Raman scattering (SERS) probes

Use of SERS probes for telomere measurement is a recently described method by Zong *et al* in 2014. Telomere and centromere SERS probes are hybridised to a genomic DNA sample. The sample is then dropped onto a SERS substrate and SERS signal is measured. If a telomere is short, fewer probes can attach and the signal

is reduced, compared to a longer telomere with greater probe attachment. The result is normalised to the centromere SERS signal. The laboratory published an updated version of the method in 2016, called SERS based *in situ* hybridisation, which allows the measurement of single telomeres (Zong *et al.*, 2016).

Quantigene chemistry

The quantigene chemistry method generates a relative telomere measurement using a hybridisation-based assay, which uses Luminex® magnetic beads with capture probes targeted to telomere repeats and a reference gene. A lysed sample is incubated with the capture probes, capture extenders, label extenders and blocking probes. Signal amplification occurs using branch DNA technology, with pre-amplifier molecules hybridised to the label extenders, multiple amplifier molecules hybridised to the pre-amplifier molecules, which provides multiple sites for biotinylated label probes. The label probes bind Streptavidin R-Phycoerythrin, producing fluorescent signal which is read on a Luminex® instrument. The signal is proportional to the amount of target DNA in the sample (Kibriya *et al.*, 2014).

4.1.1.7 3' overhang measurement methods

A 3' overhang exists on the telomere and the methods used to measure the overhang length will be described in brief for completeness. Electron microscopy was successfully used to visualise the 3' overhang and showed the presence of t-loops in the telomere overhang (Griffith *et al.*, 1999). The length of the overhang has been shown to be proportional to the rate of telomere length shortening in culture (Huffman *et al.*, 2000).

Primer/extension-nick translation (PENT)

(CCCTAA)₄ oligonucleotides are hybridised under non-denaturing conditions and extended with Taq polymerase. The products are then analysed by agarose gel electrophoresis (Makarov, Hirose and Langmore, 1997).

Telomeric-oligonucleotide ligation assay (T-OLA)

Undenatured DNA is hybridised with radioactive phosphorus (³²P) labelled (CCCTAA)_n oligonucleotides in the presence of a ligase enzyme. The products are analysed using a denaturing sequencing gel (Cimino-Reale *et al.*, 2001).

4.1.1.8 Correlation between telomere length measurement methods

Current thinking among telomere measurement experts is that, in order to check whether the qPCR method is valid, it should be compared to another method and optimised until a high R² coefficient is reached (Aviv, 2009; Aubert, Hills and Lansdorp, 2012). This thinking has arisen out of concern about the repeatability of qPCR results between laboratories (Martin-Ruiz *et al.*, 2015).

Aviv *et al* (2011) measured telomere length in 50 samples by both TRF analysis and qPCR in separate laboratories. There was a significant correlation between measurements made using the two methods. However, the R² value was improved by fitting a curvilinear model to the data compared to fitting a linear model. The inter-assay coefficient of variation for the qPCR method was higher than the TRF analysis method (6.45% versus 1.74%). Elbers *et al* (2013) compared qPCR and TRF analysis of LTL from 679 participants. The correlation between data from the two methods was only modest (R²=0.27). The inter-assay variation was higher in the qPCR measurements than the TRF measurements, 5.8% versus 1.5%, respectively. In another study, 10 different laboratories compared telomere length measurement, using qPCR and either TRF analysis or STELA in 185 samples (Martin-

Ruiz *et al.*, 2015). Again, there was a significant correlation between telomere length measurement between qPCR and TRF analysis or STELA, despite the median inter-laboratory variation for qPCR measurement being 20.7%. The inter-laboratory variation between TRF or STELA measurements was lower, at 9.2%.

4.1.2 Chapter aims & hypotheses

The primary aim of this chapter was to establish a reliable method of telomere length measurement in dog samples. The secondary aim was to see if there was a non-invasively collected sample which could be used as a reliable substitute for blood in telomere length analysis. The TRF analysis method was optimised and then used to demonstrate the reliability of a qPCR method. Once the qPCR method was established, multiple variables were analysed to see which, if any, were associated with telomere length in the individuals.

The hypotheses for the Southern blot TRF analyses were as follows: that in Labrador retrievers, buccal and leukocyte TRF would be negatively associated with age; that TRFs would be shorter in males than females; and TRFs would be shorter in entire dogs than neutered dogs. In the qPCR analyses, it was hypothesised that: leukocyte and saliva RTL would also be negatively associated with age, that saliva RTL would be shorter in males, and leukocyte and saliva RTL would be shorter in entire dogs. In addition, it was hypothesised that leukocyte RTL would: vary between breeds, be negatively associated with weight, height and cephalic index, and be positively associated with breed estimated effective population size and owner index of deprivation.

4.2 Methods

4.2.1 Samples- Bloods

Samples used in this chapter by section are summarised in Table 4-1. Blood samples were obtained from the University of Glasgow Veterinary Diagnostics Service. The service retains surplus samples for a week following receipt and then normally discards the remainder. Surplus blood samples were obtained from 14 individual Labrador retrievers for TRF analysis optimisation and 83 individual dogs of 16 different breeds for qPCR optimisation. Samples were stored at 4°C between the service performing their tests and the samples being transferred to our laboratory, at which point they were stored at -20°C until use for analysis and following this moved to -80°C storage. Information regarding age, weight, breed, clinical diagnosis, clinical pathology results and postcode were gathered from the clinical record for each animal. The postcodes were entered into the Scottish Neighbourhood Statistics website to obtain measures of deprivation for the corresponding residential area (The Scottish Government, 2015b). The estimated effective population size (EEPS) for each breed was obtained from the Kennel Club UK website (The Kennel Club Limited, 2016). EEPS was given as “n/a” for greyhound and Rhodesian ridgeback as there was increasing genetic diversity in the breeds. Figures for the cephalic index were obtained from Carrasco et al. (2014). No information on cephalic index was available for Dogue de Bordeaux or flat coated retriever. Breed height was obtained from the UK Kennel Club breed standards (The Kennel Club Limited, 2016). No information was given on height for Cavalier King Charles spaniel or Chihuahua on the UK Kennel Club website. Heights for these breeds were obtained from the American Kennel Club website (American Kennel Club, 2016). The figures for breed lifespan used in this chapter were generated using breed median age of death from three studies (Michell, 1999; Adams *et al.*, 2010; O'Neill *et al.*, 2013).

4.2.2 Samples- Labrador retriever buccal swabs

10 Labrador retriever Isohelix SK-1S (Cell Projects, Kent UK) buccal swab samples were collected by a veterinary surgeon at Abbey Veterinary Group, Paisley. The samples were picked up and DNA extraction performed on the same day of sample collection to minimise the time for bacterial growth. The date of birth and sex of the dogs were provided by the surgery.

4.2.3 Samples- Labrador retriever saliva and leukocyte DNA

Matched saliva and blood samples from Labrador retrievers were collected at the Royal Dick School of Veterinary Studies Hospital for Small Animals. Saliva samples were collected using Oragene animal kits (DNA Genotek, Ontario Canada) by Dr Dylan Clements. The samples were DNA extracted in the laboratory of Prof Kim Summers and aliquots from the DNA samples were kindly provided for this study. In addition, leukocyte DNA samples from Labrador retrievers extracted in a laboratory at the University of Manchester participating in a collaborative project with Dr Clements were generously donated.

Table 4-1 Samples used by section in Chapter 4***=University of Glasgow Veterinary Diagnostics Service**

Tissue	Source	Number	Sex	Breed	Experiment	Section
Blood	Veterinary Diagnostics*	3	1 F, 1 M, 1 MN	Labrador retriever	Optimisation of TRF analysis protocol (sample data not used for further analyses)	4.3.1
Blood	Veterinary Diagnostics*	20	5 F, 8 FN, 1 M, 6 MN	Labrador retriever	Optimisation of qPCR protocol (sample data not used for further analyses)	4.3.2
Blood	Veterinary Diagnostics*	14	3 F, 6 FN 1 M, 4 MN	Labrador retriever	Checking TRF analysis protocol	4.3.4
Buccal	Abbey Veterinary Group	10	5 F, 2 FN, 1 M, 2 MN	Labrador retriever	TRF analysis of buccal samples	4.3.5
Blood	Veterinary Diagnostics*	83	52 M, 31 MN	Mix of 16 breeds	Checking qPCR protocol 14 of these samples analysed using both qPCR and TRF methods to check if results correlated	4.3.6
Blood & saliva	University of Edinburgh	24	2 F, 7 FN, 3 M, 12 MN	Labrador retriever	qPCR analysis of paired tissue samples	4.3.7
Blood	University of Manchester	50	16 F, 11 FN, 19 M, 4 MN	Labrador retriever	Further qPCR samples	4.3.8

4.2.4 DNA extraction

DNA was extracted from blood a using a column method (section 2.2.2.1). 40 blood samples were extracted using a QIAamp Blood Midi kit (Qiagen, Manchester UK), the remaining 43 qPCR optimisation and the 14 TRF optimisation blood samples using a Nucleospin Blood Mini kit (Macherey Nagel, Düren Germany). A midi kit was used in a subsection of samples to attempt to obtain higher DNA yields for Southern blotting purposes. To obtain sufficient DNA for TRF analysis, buccal swabs were extracted using a phenol-chloroform based method (section 2.2.2.2). DNA quantity and quality were measured using spectrophotometry (section 2.2.5).

4.2.5 Data analysis

Normality checks were performed on data using a Shapiro-Wilk test. A p value of <0.10 resulted in the variable being treated as non-parametric. If possible, non-parametric data were transformed to a normal distribution, using a natural log transformation, and a subsequent Shapiro-Wilk test performed to justify an assumption of normality for the transformed data. Pairwise comparisons were performed using t-tests (normally distributed data) or Wilcoxon rank-sum tests (non-normal data). One-way analysis of variance (normal data) or Kruskal-Wallis (non-normal data) tests were used to compare values when more than two groups were present, for example comparison of telomere length between breeds. To test the association between an explanatory variable and a non-parametric continuous response variable, Spearman's rank correlation was used, for example when exploring the association between dog weight and leukocyte count.

To analyse the association between a single continuous explanatory variable and a normally distributed response variable, linear models were used, except in comparing qPCR measures to TRF measures, for which a quadratic regression was also employed by using the “poly” function in R. Linear models were also used to analyse associations between multiple explanatory variables and a normally distributed response variable. Model residuals were checked by using the `plot(modelname)` function in R. Where several explanatory variables were available, all explanatory variables were included and the “drop1” function used

to assess which explanatory variables, if any, to remove. There were no instances in which associations between multiple explanatory variables and a non-normal response variable needed to be tested. If the intercept was not statistically significant ($p < 0.05$), the model constant was suppressed.

All analyses were performed in R (R Core Team, 2014). Statistical significance level was taken as $p < 0.05$.

4.3 Results

4.3.1 Southern Blot

Figure 4.1 describes the overall steps of a telomere restriction fragment length measurement by Southern blot analysis. In this section, each step will be described in further detail and, where appropriate, how the protocol was optimised will be detailed. As described in section 2.2.6, prior to starting this protocol, the DNA integrity of each sample was checked by gel electrophoresis. The protocol was based on the manual provided with the kit used for the procedure: the TeloTAGGG Telomere Length Assay (Roche), with amendments inspired by Kimura *et al* (2010). All buffer recipes are detailed in section 2.1.3.

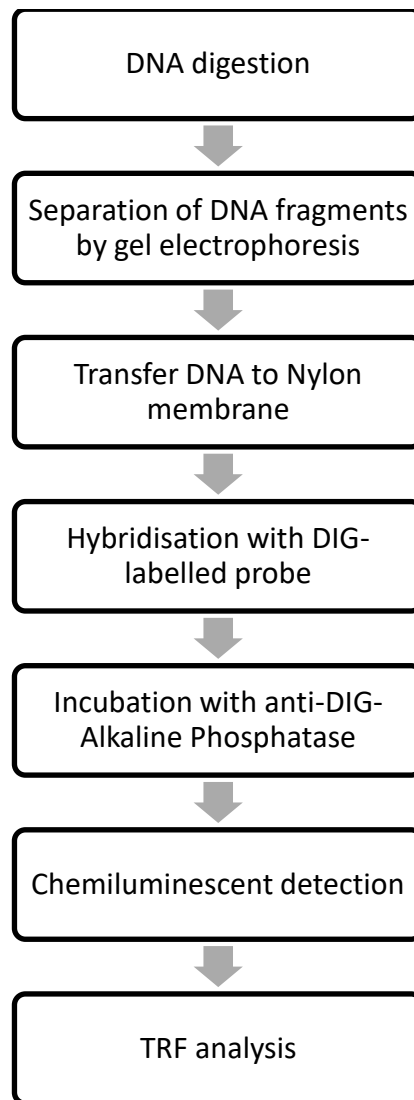


Figure 4.1 Workflow of Southern blot analysis for measurement of telomere restriction fragment length

4.3.1.1 DNA Digestion with restriction endonucleases

The DNA is cut using restriction enzymes which do not have cutting sites in the telomere, but cut frequently in non-telomeric DNA. Adjacent to the telomere is the subtelomeric region which consists of non-canonical repeats (Allshire, Dempster and Hastie, 1989) and the choice of restriction enzymes determines whether this region is cut. The uncut section is referred to as the TRF. The most commonly used combination of enzymes is *HinfI* and *RsaI*, and these were used in this study. These enzymes do not cut in the subtelomeric region. Another combination used in telomere studies, *HphI* and *MnII*, results in cuts in the

subtelomeric region and has been shown to result in TRF lengths that are approximately 1-1.5 kb shorter than when *Hinfl*/*RsaI* are used (Baird *et al.*, 2006; Hunt *et al.*, 2008). One of the criticisms of Southern blots, and particularly of the use of *Hinfl*/*RsaI*, is that the subtelomeric region is variable in length between individuals, resulting in different TRF lengths (Cawthon, 2002). The combination of *Hinfl*/*RsaI*, however, is not only the most commonly used in human studies, but has been used in multiple studies of dog telomeres and therefore the use of this enzyme combination in this thesis would enable results to be comparable to the limited dog telomere literature (Nasir *et al.*, 2001; McKeivitt *et al.*, 2002; Cadile *et al.*, 2007; Benetos *et al.*, 2011).

For each sample, in a 0.5ml microcentrifuge tube, 1µg of DNA was diluted with nuclease free water to a final volume of 17µl. In addition, a tube was set up containing 1µg “Control-DNA” using the control provided in the TeloTAGGG® kit. 2µl of digestion buffer, 0.5µl *Hinfl* (20 units) and 0.5µl *RsaI* (20 units) were added to each sample and mixed. The tubes were incubated in a 37°C heat block for 16 hours and, after this time, the reaction was stopped by adding 5µl of 5X gel electrophoresis loading buffer and briefly centrifuging the tubes. If not used immediately, the tubes were stored at -20°C.

4.3.1.2 Gel Electrophoresis

The DNA fragments were separated by agarose gel electrophoresis. Initially, a 0.8% 15cm length agarose gel was used. Later on, a 0.5% 20cm gel was used to improve separation and measurement of longer telomeres. The agarose percentage used is indicated for each experiment run. A DIG labelled molecular weight marker was run alongside the samples on the gel. To prepare the marker for loading, 4µl of DIG molecular weight marker was added to 12µl nuclease-free water and 4µl of 5X loading buffer in a 0.5ml microcentrifuge tube, which was then briefly centrifuged. 10µl of molecular weight marker mix was added to the two outer wells in the gel. The digested DNA samples were loaded in the remaining wells and the gel run. The 0.8% was run at 75V and the 0.5% gel was run

at 40V. The gels were run until the blue tracking dye was 2cm from the end of the gel, which took approximately 5 hours for a 0.8% gel and 20 hours for the 0.5% gel.

4.3.1.3 Transfer

Prior to transferring the DNA to a nylon membrane, the gel was depurinated, denatured and neutralised. The gel was placed in a sterile container and submerged in 0.25M hydrochloric acid and agitated on a shaker, until the bromophenol blue on the gel changed colour to yellow. This took approximately 10 minutes to occur. The gel was rinsed twice with water, then submerged in denaturation solution twice, 15 minutes each time with agitation. Once again, the gel was rinsed twice with water and then finally submerged in neutralisation solution twice for 15 minutes each time with agitation.

A piece of nylon membrane was cut to the same size as the gel, submerged in water and then in 2X SSC for 10 minutes. The membrane was handled with clean forceps only. The transfer equipment was then prepared as described by Brown (2001). A solid support was placed in a large Pyrex dish, with wicks made of Whatman 3MM filter paper. 20X SSC was poured into the dish so the ends of the wick were submerged in buffer. 3 pieces of Whatman 3M paper were cut to the same size as the solid support, placed on top of the wick and wetted with 20X SSC. The gel was placed on the filter paper and air bubbles removed by rolling a glass pipette over the surface. Plastic wrap was placed over the edges of the gel to ensure the buffer flowed through the gel rather than around it. The pre-soaked membrane was then placed on top of the gel, again removing any air bubbles with a pipette. 5 sheets of Whatman 3MM paper, cut to the same size as the membrane, were placed on top. Paper towels were then stacked on top of the filter paper, to a height of approximately 10cm, and a glass plate and weight used to hold everything in place. This was then left overnight to allow time for the transfer to take place. The next day, the stack was disassembled. The membrane was washed briefly with 2X SSC and then placed on Whatman 3M paper. The DNA

was fixed by UV-crosslinking using 120mJ of energy and then the membrane washed twice with 2X SSC, for 15 minutes each time with agitation.

4.3.1.4 Hybridisation

The first time the experiment was run, the membrane was placed in a hybridisation tube, 18ml of prewarmed to 42 °C DIG Easy Hyb Granules was added and the tube capped. The tube was placed in a hybridisation oven and rotated for one hour at 42 °C. Following this, the prehybridisation solution was removed and the hybridisation solution was added to the membrane for three hours at 42 °C. The hybridisation solution was made by adding 1.5µl DIG (digoxigenen) labelled Telomere probe to 7.5ml DIG Easy Hyb Granules. Using these conditions, the first film which was generated at the end of the whole experiment had a very high background optical density (Figure 4.2). As a result, the probe concentration and time spent pre-hybridising the blot were optimised to reduce the background in future experiments. In previous studies, probes have been labelled with radio isotopes such as ^{32}P rather than DIG as used here. Use of these probes are said to improve the detection sensitivity, however they come with safety risks which outweigh the added precision obtained (Saldanha, Andrews and Tollefsbol, 2003).

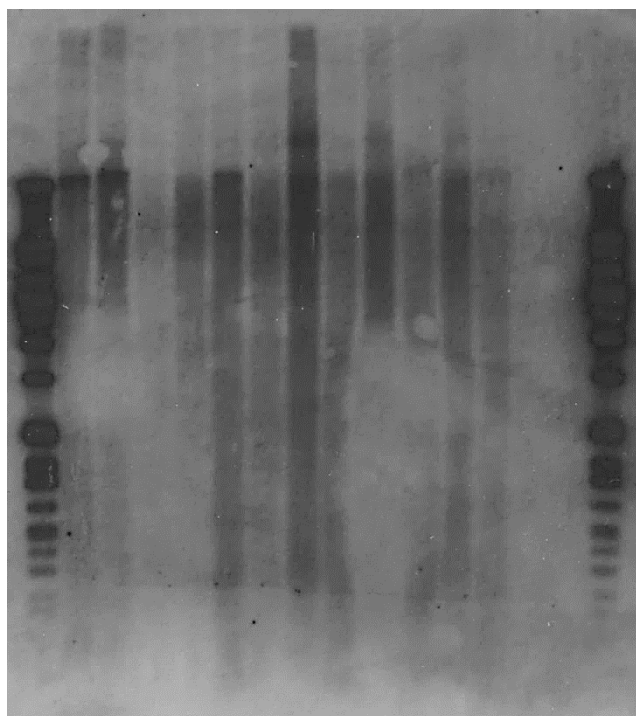


Figure 4.2 First Southern blot conducted during experiment set up with a very high background optical density

DNA was extracted from three Labrador retriever blood samples using a column extraction as described in section 2.2.2.1 for use in the optimisation experiments. The blood samples were surplus blood from the University of Glasgow Veterinary Diagnostics Service. Sample 1 was from a 2 year old entire female, sample 2 was from a 7 year old neutered male and sample 3 was from a 12 year old entire male. 4 sets of digests of the 3 samples were set up and digested overnight, the gel run and blot transferred as described previously. The membrane was cut into four, with one set of the samples on each strip of the membrane. Two of the strips had one hour of prehybridisation at 42°C and the other two had the prehybridisation solution left on overnight again at 42 °C. Two concentrations of hybridisation solution were made up: one with 1µl of Telomere probe in 7.5ml of DIG Easy Hyb Granules and one with 1µl of Telomere probe in 10ml of DIG Easy Hyb Granules. Figure 4.3 shows the resulting blots. No improvement in background reading was observed by having an overnight prehybridisation stage versus a one-hour stage, so a one hour prehybridisation was subsequently used. There was still a high background optical density with both 1µl probe in 7.5ml and 1µl probe in 10ml so further experiments were set up with 1µl probe in 12.5ml and 1µl probe in 15ml

(Figure 4.4). Rather than performing new digests, two of the blots were stripped by washing the blot in stripping buffer at 37°C in a hybridisation oven with rotation twice for 15 minutes each time. The blot was then rinsed in 2X SSC. A film was developed from the blot to check the stripping procedure had been successful, then the protocol started from the one hour prehybridisation stage. 1µl probe in 15ml granules resulted in a very low band optical density reading and required a long film exposure time to visualise the bands, therefore 1µl probe in 12.5ml granules was decided upon for future experiments.

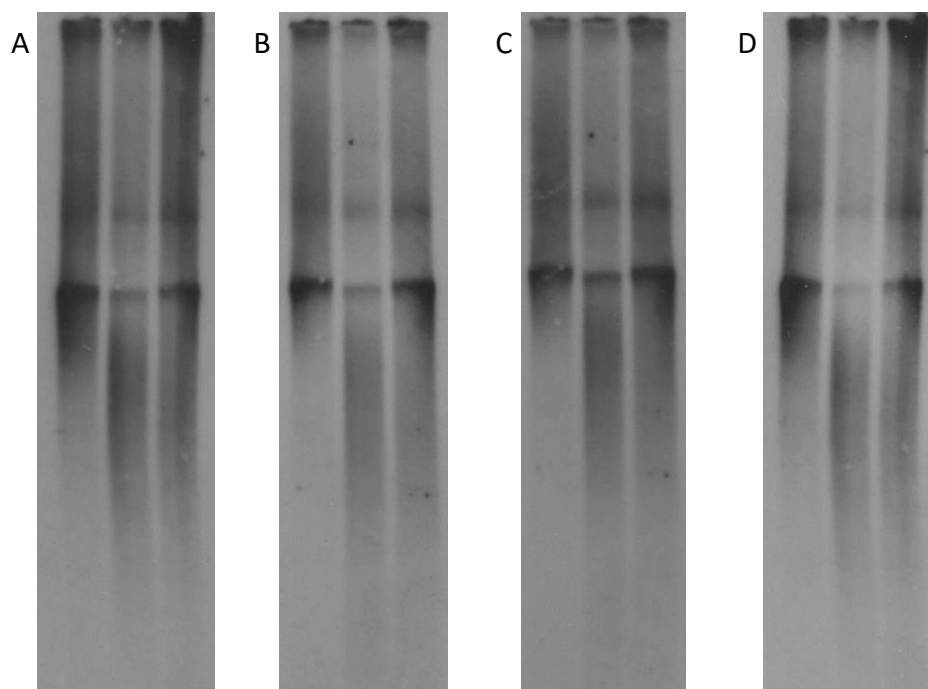


Figure 4.3 Southern blots varying the prehybridisation time and probe concentration with the same 3 Labrador retriever blood DNA samples digested in each blot. 2 blots had a 1 hour prehybridisation (A and C) and 2 had an overnight prehybridisation (B and D). The hybridisation buffers were either made using 1µl Telomere probe in 7.5ml hybridisation granules (blots A and B) or 1µl Telomere probe in 10ml hybridisation granules (blots C and D).

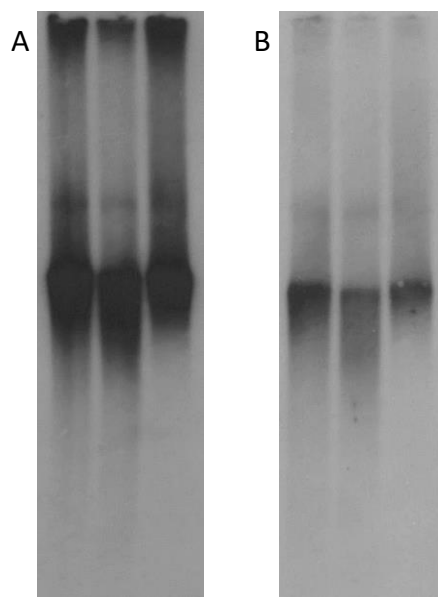


Figure 4.4 Southern blots comparing telomere probe concentration

1 μ l probe in 12.5ml hybridisation granules (A) and 1 μ l probe in 15ml hybridisation granules (B). Note that the dark bands in blot A are a result of a long film exposure time, not an excess of probe.

Following 3 hours of hybridisation with a Telomere probe, the solution was discarded and then the membrane was washed twice with stringent wash buffer I at room temperature on a shaker for 5 minutes each time. Following this, the membrane was put back in the hybridisation oven at 50°C and washed twice with stringent wash buffer II with rotation, each wash for 20 minutes.

4.3.1.5 Anti-DIG-Alkaline Phosphatase incubation

All the steps in this section were performed at room temperature on a rocker. The membrane was washed with 100ml washing buffer for 5 minutes, then incubated in 100ml blocking solution for 30 minutes. Following this the membrane was placed in 75ml of Anti-DIG-AP solution for 30 minutes. Two 15 minute washes were then performed using 100ml of washing buffer each time.

4.3.1.6 Chemiluminescent detection

The membrane was incubated in 100ml detection buffer for 5 minutes on a rocker at room temperature, then the detection buffer discarded and the membrane placed on a sheet of Whatman 3MM paper, DNA side up, to remove excess liquid. The membrane was placed in a hybridisation bag and 3ml of substrate solution applied to the DNA side of the membrane. The substrate solution contains CDP-Star, which is metabolised by the alkaline phosphatase in the Anti-DIG-AP solution and produces a visible signal. The upper sheet of the bag was placed on the DNA side of the membrane and air bubbles removed by rolling a pipette over the bag. After a 5-minute incubation period, excess substrate solution was squeezed out of the bag and the edges were heat sealed. The membrane was then exposed to X-ray film for approximately 5 minutes and the film developed. Timing of exposure was adjusted and multiple films exposed until the optimum film was produced for each membrane.

4.3.1.7 TRF analysis

The film was scanned using a flatbed scanner and saved as a .jpg file. TotalLab version 13.01 software (TotalLab Ltd UK, 2013) was used to analyse the images. The .jpg file was opened in TotalLab and saved as a .tiff file. Line selections were composed using the draw shape tool for each sample lane. The software then generated plots of optical density versus DNA migration distance. The data were exported to a Microsoft Excel file. The distance that each band of the molecular weight marker had migrated was plotted against the weight of that band. Using R statistical software, third order log-linear regression was fitted for the molecular weight migration. The resulting equation was used to convert the sample migration distances to molecular weight. A background optical density reading using an area outside of the lanes was subtracted from all readings. Mean TRF was calculated for each sample using the formula:

$$\text{Mean TRF length} = \frac{\sum(\text{OD}_i \times \text{MW}_i)}{\sum \text{OD}}$$

where OD_i is the optical density at position i and MW_i is the molecular weight at position i

Using this formula, an average length of TRFs is reported for each sample. By examining the film, one can also get an idea of the spread of lengths. However, short telomeres are often hard to visualise as fewer probes are hybridised to them, so some information may be missed (Aubert, Hills and Lansdorp, 2012). Duplicate analyses were carried out on each DNA sample on different gels to account for inter-gel variation.

4.3.2 qPCR Optimisation

4.3.2.1 Protocol optimisation

The telomere length qPCR assay was developed as a means of finding a simple, fast method of telomere measurement. It had previously been presumed that a PCR based assay would not be possible due to the generation of primer-dimer products with the use of primers designed to hybridise to the TTAGGG and complementary repeats. Cawthon (2002) therefore developed a pair of primers to overcome this issue. The key characteristic of the primers was that they were designed to amplify only when annealed to a telomere hexamer, and not when hybridised to the other primer.

The telomere primers were not a perfect match with the target sequence, but could hybridise with partially complementary sequences along the length of the telomere in both directions. The 5' end of the primers could not hybridise to the sequence. The complementary products to this 5' end were generated at the 3' end of products completed in each PCR cycle. This blocked 3' ends from initiating DNA synthesis in subsequent cycles from telomere amplification products.

The initial primer design by Cawthon was modified by Epel *et al.* (2004) and this new design was successfully applied to the measurement of dog telomeres by Fick *et al* (2012). Therefore, this modified design was selected for use in this thesis.

The sequences were as follows:

Tel1b primer (forward): CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT

Tel2b primer (reverse): GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT

A representation of the primers binding to the target is depicted in Figure 4.5A, and potential primer-primer bonding in Figure 4.5B, modified from Cawthon (2002) to reflect the updated primer design.

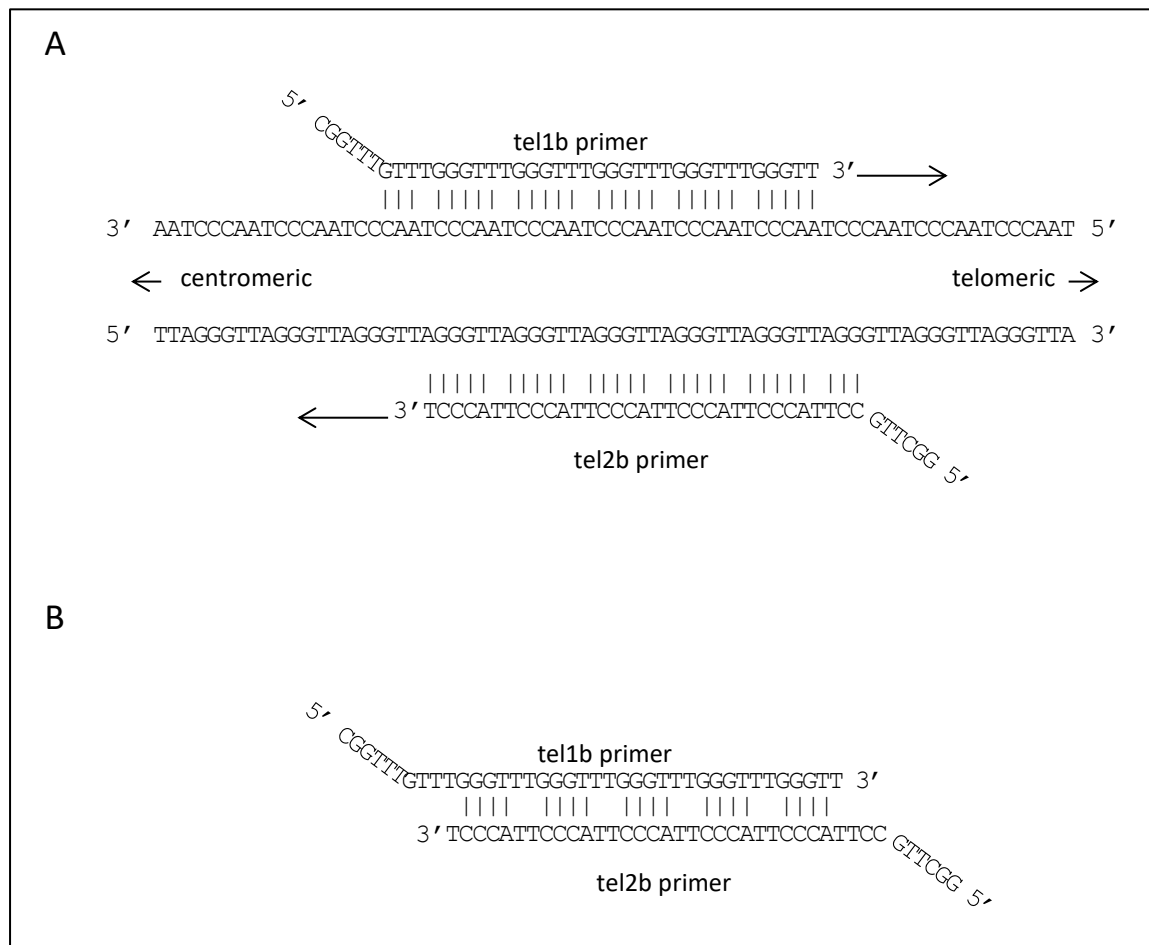


Figure 4.5 Annealing of primers in telomere DNA in the first round of PCR and primers annealing to each other

A- Annealing of primers in telomere DNA in the first round of PCR, arrows at 3' end of primers represent direction in which bases are added. There are base mismatches between the primers and template DNA every sixth base. B- Primers annealing to each other, there are two base mismatches after every four matches. The last base at the 3' ends of the primers cannot form a stable pair with the base opposite, preventing the addition of bases.

The first step of the optimisation of the telomere protocol was to determine the concentration of the telomere primers. Primer concentrations are seen as optimal when there is a low variability between replicates, adequate signal to noise ratio and no primer dimers seen (Mikeska and Dobrovic, 2009). An “optimisation matrix” of primer concentrations was used (as in Nolan *et al.* 2006) to check several combinations of forward and reverse primer concentrations. An example matrix is shown below:

	Forward primer concentration (nM)				
Reverse primer concentration (nM)		100	200	300	400
	100	100 F/100 R	200 F/100 R	300 F/100 R	400 F/100 R
	200	100 F/200 R	200 F/200 R	300 F/200 R	400 F/200 R
	300	100 F/300 R	200 F/300 R	300 F/300 R	400 F/300 R
	400	100 F/400 R	200 F/400 R	300 F/400 R	400 F/400 R

The primer concentration matrices were run both with 10ng DNA and a water blank to look at what concentration primer dimers may occur. This level of checking for primer dimers is not usually performed, however it was justified here because the telomere primers may have been more prone to have this issue than other sets of primers. Forward and reverse primers at the appropriate volume to produce the required concentrations were added to 10µl Absolute blue® SYBR green low ROX mix, 10ng DNA if required and then ddH₂O added to a final volume of 20µl per well. A wider range of primer concentrations were used initially, then a narrower range to get the primers concentrations required to the nearest 50nM. The optimum primer concentrations using this method were 100nM tel1b and 100nM tel2b.

This process was then repeated for the reference gene primer set. The reference gene initially selected was 36B4, the gene encoding for acidic ribosomal phosphoprotein PO, and primer sequences used as per Fick *et al* (2012). However, persistent contamination was seen in the no template control wells despite several attempts to rectify this. Therefore, new primers were designed using CLC Genomics Workbench v6.0.4 (CLC Bio, Denmark). The gene selected was haemoglobin delta (HBD), which is located on chromosome 21. Haemoglobin genes have previously been used in telomere studies (e.g. Guzzardi *et al.* 2015) and HBD has the refseq status of “model” on the NCBI website, which is not the case for all dog genes. The sequences of the HBD primers were:

HBD Forward: CAGTGCTGAGGAGTCCATAC

HBD Reverse: AAGACCCAACTCATGATGTCC

The concentrations of the HBD primers were then optimised using a matrix as described previously. Using this method, the concentrations decided upon were 100nM forward, 150nM reverse.

For the absolute quantification of telomeric DNA in each sample, standard curves using oligomer standards needed to be included on each plate. Oligo standards were polyacrylamide gel purified and the sequences of the telomere and HBD standards were:

Telomere standard:

(TTAGGG)¹⁴

HBD standard:

CAGTGCTGAGGAGTCCATACTTATGCAACTAGAACTTCCTTTACACTCTGAACAA
CATCCTGATGCCCATACCCTTTACGGACATCATGAGTTGGGTCTT

To each standard well, 10ng of pBR322 DNA was added as background DNA. Rolling circle amplification (RCA) was used to amplify pBR322 plasmid DNA (Sigma Aldrich, Gillingham UK) in order to generate sufficient background DNA for multiple standard curve experiments. 1µl of pBR322 was added to 4µl PCR grade H₂O in a 0.2ml microcentrifuge tube. By placing the tube in a thermocycler at 95°C for 3 minutes, the DNA was denatured. The tube was then placed on ice. 4µl of 25µM dNTP, 2.75µl of 50µM random hexamer, 0.5µl Phi29, 10µl reaction buffer, 2µl BSA, and 75.75µl ddH₂O were added to the denatured DNA. The tube was returned to the thermocycler, which was then run at 30°C for 18 hours, followed by 65°C for 10 minutes. The concentration of the resulting products was measured using spectrophotometry.

The quantities of oligomer standards in the standard curve dilutions needed to be optimised such that all dilutions were within the linear range of the assay, and test sample C_T s fell approximately in the middle of the curve. The C_T is the PCR cycle where there is sufficiently greater fluorescent signal than the background to cross a threshold, as described in section 2.2.6. The starting standard curve amounts were based on Fick et al (2012), with the telomere standard curve starting with $60 \times 10^{-12} \text{g}$ per reaction, followed by five further one in ten dilutions. The HBD standard was based on the DNA quantities required for the reference gene in the same paper, with six dilutions starting at $200 \times 10^{-12} \text{g}$ and ending in $2 \times 10^{-15} \text{g}$. The final point on the telomere standard curve was beyond the linear limit of the reaction, and therefore the standard curve was started at a higher amount. The converse was true for the HBD curve, and the amount at which the dilution series was started was reduced.

Once the amounts in the standard curves were decided upon, the qPCR was run using a range of annealing temperatures to check what temperature resulted in an efficiency closest to 100%. Annealing temperatures tested depended on the melting temperature of the primers. Between 58°C and 63°C were tested for the telomere reaction, and temperatures between 50°C and 55°C for the HBD reaction. The annealing temperature was adjusted to 59°C for the telomere reaction and 52°C for HBD. The final cycling conditions were decided upon once the sample DNA quantity was optimised. A series of template amounts were run, between 5ng and 75ng, to see what amount would result in the C_T of the samples falling in the middle of the curve. When 25ng of sample was used, the vast majority of sample C_T s fell around the middle of both curves. As a result, 25ng was decided upon, and the amount of pBR322 in the standard curve wells was also changed to 25ng to reflect this.

Both telomere and HBD PCR began with a 95°C 15-minute incubation to activate the Thermo-Start™ DNA polymerase. The telomere thermal cycling profile then

consisted of 28 cycles of 95 °C for 15 seconds, 59 °C for 30 seconds and 72 °C for 30 seconds. Following the 15-minute incubation, the HBD cycling profile had 32 cycles of 95 °C for 15 seconds, 52 °C for 30 seconds and 72 °C for 30 seconds. After these cycles, melt curves were run for both PCRs (60-95 °C). All PCRs were performed on an Applied Biosystems 7500fast machine using machine compatible white 96 well plates (Starlab, Milton Keynes UK). White plates were used instead of clear plates as white plates have been shown to increase qPCR sensitivity due to increased fluorescence reflection compared to clear plates (Reiter and Pfaffl, 2008). Mastermixes were made for the telomere and HBD plate for set up, 15µl of which was added to each well and 5µl of test sample, ddH₂O (for no template control wells) or standard with pBR322 added to the appropriate wells. The mastermixes consisted of Absolute blue® SYBR green mix (final concentration 1X) HBD or telomere primers depending on the plate (final concentrations 100nM Tel1b/100nM Tel2b or 100nM HBDF/150nM HBDR) and ddH₂O. The plates were sealed with Microamp optical cap strips and briefly centrifuged. The plates were protected from light as much as possible, as SYBR green is light sensitive. DNAZap® was used prior to setting up experiments to clean surfaces and pipettes, to remove potentially contaminating nucleic acids.

4.3.2.2 Absolute telomere length calculation

Once the final quantities were decided upon for the standard curve points, the standard curve calculations were performed as per O'Callaghan et al (2008) with adjustments.

The number of telomere repeats in each standard was calculated using the following:

- The oligomer standard had a molecular weight of 26667.2.
- The weight of one molecule was $26667.2 / 6.02 \times 10^{23}$ (Avogadro's number) = $0.44 \times 10^{-19} \text{g}$

- The highest concentration of telomere standard had 60pg per reaction. Therefore, there were $60 \times 10^{-12} \text{g} / 0.44 \times 10^{-19} \text{g} = 1.36 \times 10^9$ molecules in Tel standard 1.
- The amount of telomere sequence in Tel standard 1 was $1.36 \times 10^9 \times 84 (\text{oligomer length}) = 1.18 \times 10^{11}$ bases or 1.18×10^8 kb.

A standard curve was performed through 1 in 10 serial dilutions of Tel standard 1 to create 6 standards. The standard curve was used to measure the amount of telomere sequence per sample in kb. In addition, a standard curve was created for 6 HBD standards as per the telomere standard curve and was used to measure the number of diploid genome copies in each sample.

Genome copy number calculations were as follows:

- The synthesised HBD oligomer standard was 102 bases long with a molecular weight of 31239.3.
- The weight of the HBD standard was $31239.3 / 6.02 \times 10^{23} = 0.5189 \times 10^{-19} \text{g}$
- HBD standard 1 had $2 \times 10^{-12} \text{g}$ per reaction. Therefore, there were $2 \times 10^{-12} \text{g} / 0.5189 \times 10^{-19} \text{g} = 3.85 \times 10^7$ copies of oligomer in HBD standard 1.
- As there are two copies of HBD per diploid genome, HBD standard 1 is equivalent to 1.93×10^7 diploid genome copies.

The standard curves were required to have $100 \pm 10\%$ efficiency and R^2 value of the standard curve to be at least 0.98. Example standard curves are shown in Figure 4.6 and Figure 4.7. The amount of telomere sequence and genome copies per sample were obtained using the standard curve equations. To obtain the telomere length per diploid genome, the amount of telomere sequence was divided by the number of diploid genome copies. This number can be further divided by 156 (there are 78 chromosomes per dog diploid genome) to obtain average individual telomere length, however in the original method paper and others the telomere

length per diploid genome is used for data analyses (O'Callaghan *et al.*, 2008; Izzo *et al.*, 2011; Barrett *et al.*, 2012) and so this tradition was followed here.

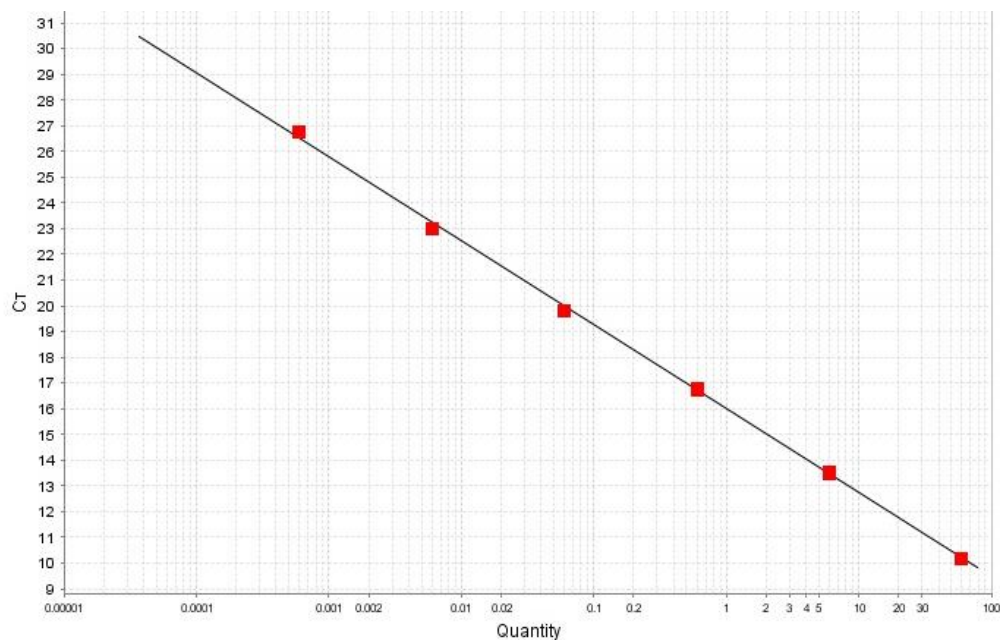


Figure 4.6 Example telomere standard curve

$R^2=0.999$. The slope here had a gradient of -3.26, equating to an efficiency of 102.7%.

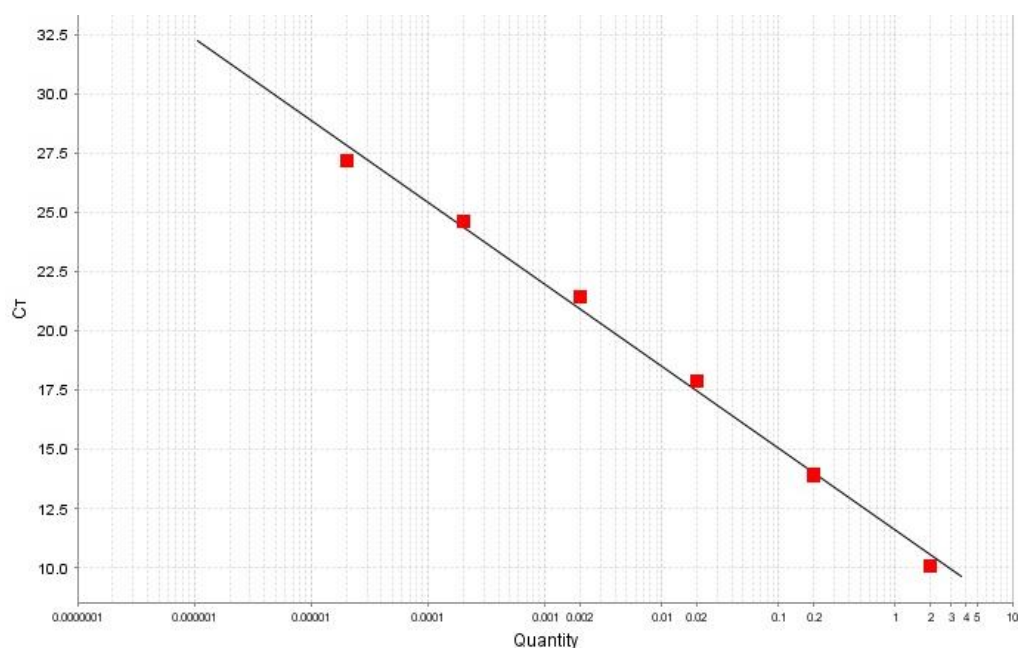


Figure 4.7 Example HBD standard curve

$R^2=0.995$. The slope here had a gradient of -3.462, equating to a 94.5% PCR efficiency.

20 Labrador retriever leukocyte DNA samples were used to test the absolute telomere length (aTL) protocol. The resulting values for telomere length per diploid genome ranged between 571kb and 4469kb. When the experiment was repeated, the values ranged from 337kb to 7646kb. These values were higher and over a wider range than anticipated. For example, in a study using human leukocyte DNA, aTL in kb per diploid genome ranged between 35 and 260 (O'Callaghan and Fenech, 2011). In a study of Australian sea lions using fin biopsy DNA, aTL per diploid genome ranged from 16.49 to 379.85kb (Izzo *et al.*, 2011). Measurements made in Seychelles warbler erythrocyte DNA were closer in length to those measured here: kb per diploid genome ranged from 1172 to 4737 (Barrett *et al.*, 2012). Fick *et al.* (2012) calculated the individual telomere length, and the measures varied from less than 10kb to greater than 50kb (actual range not quoted in the paper), which would equate to a telomere length per diploid genome of less than 1560 to over 7800, similar to the figures found here, however these measurements significantly declined with age, whereas no significant relationship was found with age in the test samples ($R^2=0.001$, $p=0.87$). The measurements made here would indicate that the dogs with the longest aTL had telomere lengths 30 times longer than that found by O'Callaghan & Fenech (2011). This would not be in keeping with the difference between published canine and human TRF measurements, which have shown that canine LTL is approximately twice the length of human telomere length. TRF lengths of approximately 16kb were measured in human foetal cord blood (Counter *et al.*, 1995), a mean of 7.5kb in leukocytes from 635 adults aged 20 to 40 (Aviv *et al.*, 2009) and 5kb in leukocytes from human centenarians (Vaziri *et al.*, 1993), compared to 9.7 to 23kb TRF length in dog leukocyte samples (Nasir *et al.*, 2001; McKeivitt *et al.*, 2002). In addition, 7646kb aTL per diploid genome would equate to an individual telomere length of 49kb. O'Callaghan *et al.* (2008) found that aTL measurements to be shorter than TRF measurements by approximately 7kb in humans, which they state reflects that TRF analysis measurements include the region proximal to the telomere. Therefore, 49kb seemed improbably long.

4.3.2.3 Relative telomere length calculation

Given the inter-assay variability, the query over the measured lengths, and the wide range of measurements obtained, it was decided to switch to the more commonly used relative telomere length method. The telomere length using this method is given as a relative measure to a selected reference sample run on every plate, using the formula:

$$\begin{aligned} & \text{relative telomere length} \\ &= 2^{-[(C_t^{\text{telomere sample}} - C_t^{\text{HBD sample}}) - (C_t^{\text{telomere control}} - C_t^{\text{HBD control}})]} \end{aligned}$$

The formula includes 2 to the power of the differences in C_T as the product of the reaction doubles with each PCR cycle, as described in section 2.2.6.

Initially, MDCK cell line DNA was used as the reference sample, as large quantities of DNA could be easily obtained, so there would be sufficient DNA for multiple experiments. All blood samples tested had a longer relative telomere length (RTL) than the MDCK DNA, and the RTL values averaged at 8.80. This equates to the telomere length of the samples averaging at 8.8 times the length of MDCK. For this reason, the author was given advice to change the reference sample by Prof P Shiels (personal communication).

To make a reference sample with sufficient DNA to be used in every experiment, and a similar telomere length to the leukocyte DNA samples, 20 canine blood samples from dogs of various breeds and ages (surplus blood from VDS) were combined and DNA extracted using a Qiagen maxi column (Manchester, UK). A second multi sample DNA extraction was performed to create a sample to use as an inter-assay control. MDCK DNA was also used as an inter-assay control sample. The RTL of both samples was calculated each time the assay was performed to check for variability between runs.

A standard curve was still run on each plate, as per the aTL method, to check the efficiency of each qPCR. However, for the RTL method, the standard curve was made up of five serial dilutions of reference DNA, with amounts of DNA at each point such that the midpoint of the curve has the same amount of DNA as used for the test samples. The standard curve points used were 100ng, 50ng, 25ng, 12.5ng and 6.25ng. The 25ng point is then used as the reference sample relative to which all other telomere lengths are measured.

The plate layout used for each experiment is shown in Figure 4.8. Two plates were run for each experiment set: one using telomere primers, and one using HBD primers (used to adjust for variation in quantity of DNA in each well in the telomere plate). Each sample, control, and standard curve point was run in triplicate. The mean C_T value of the three wells was used for data analyses. The percentage coefficient of variation (% CV) was calculated for each triplicate repeat, by dividing the standard deviation by the mean and multiplying by 100. If the % CV was 5 or above, the sample was repeated, provided sufficient DNA was available. If insufficient DNA remained, the sample was excluded from data analyses.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	1	1	9	9	9	17	17	17	S1	S1	S1
B	2	2	2	10	10	10	18	18	18	S2	S2	S2
C	3	3	3	11	11	11	19	19	19	S3	S3	S3
D	4	4	4	12	12	12	20	20	20	S4	S4	S4
E	5	5	5	13	13	13	21	21	21	S5	S5	S5
F	6	6	6	14	14	14	22	22	22	C1	C1	C1
G	7	7	7	15	15	15	23	23	23	C2	C2	C2
H	8	8	8	16	16	16	24	24	24	NTC	NTC	NTC

Figure 4.8 96 well plate set up for qPCR experiments

24 samples were run in triplicate on each plate. S= Standard (made using serial dilutions of a sample made using 20 blood samples), C=control (C1= second sample made using 20 blood samples, C2= MDCK). NTC= No template control.

4.3.3 TRF analysis optimisation samples

Blood samples from the VDS were used to check the optimisation of the TRF analysis protocol. All dogs were Labrador retrievers to remove any breed effect on telomere length. Subsequently to this, a set of buccal swabs from Abbey Veterinary Group, Paisley were used to see if the protocol could be applied to buccal cell DNA. 0.8% agarose gels were used in these experiments. Buccal swabs were DNA extracted using a phenol-chloroform based method, as preliminary experiments showed that using a column based extraction resulted in a DNA yield ranging from 550ng to 5590ng, with a mean of 1570ng, with a concentration ranging from 5.5 to 55.9ng/ μ l. The minimum requirement was a concentration of 55.8ng/ μ l (total of 1 μ g). Therefore, many samples extracted using a column method did not produce sufficient DNA to perform TRF analysis. Phenol-chloroform based extraction method resulted in an average DNA yield of 12.6 μ g per swab, with an average concentration of 126.2ng/ μ l. The 260/230 absorbance ratios were lower for the phenol-chloroform samples than the column extracted samples.

4.3.4 Labrador retriever leukocyte TRF analysis

14 Labrador retriever leukocyte DNA samples were used for TRF analysis. There were 4 male neutered, 1 male entire, 6 female neutered and 3 female entire dogs, whose ages ranged between 0.6 years and 13.4 years. The median age was 7.87 years. One sample failed the agarose gel quality check and was excluded from further analysis. TRF measurements ranged from 8.1 to 14.9kb, with a mean of 11.0kb. Figure 4.9 shows the linear regression of TRF and age, which was not statistically significant ($F=3.17_{(1,11)}$, $R^2=0.22$, $p=0.10$).

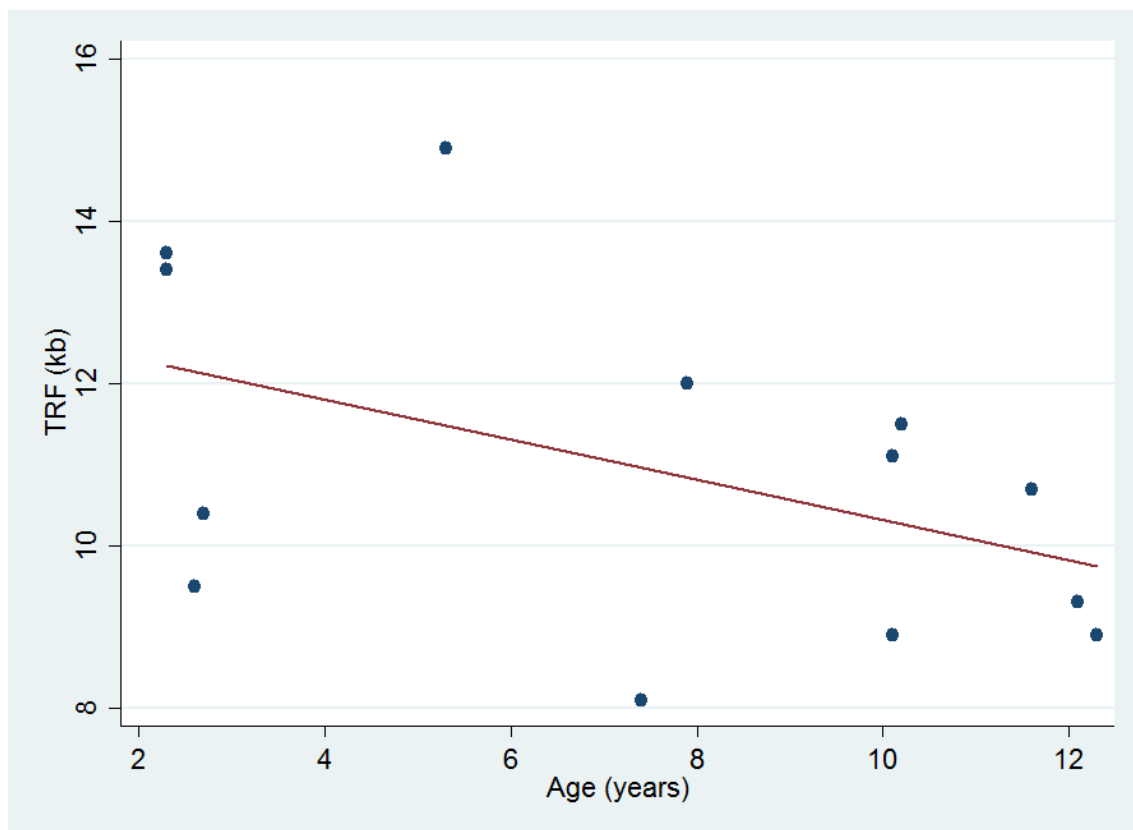


Figure 4.9 Leukocyte TRF by dog age

Scatter plot of leukocyte TRF by age of a group of Labrador retrievers, with regression line. The linear model was not statistically significant ($R^2=0.22$, $p=0.10$). $N=13$.

Factors which may have modified the association between TRF length and age were then considered to explore whether there was a reason that a significant negative correlation was not generated, aside from the sample size. Firstly, sex was considered. In females, the mean TRF length was 11.6kb, compared to 9.5kb

in males (Figure 4.10A). Using a t-test, this difference was found to be statistically significant ($p=0.04$). The median age of females was less than that of males, 5.3 compared to 9, but this difference was not significant (Wilcoxon rank sum test, $p=0.31$). There was no significant difference in TRF length between neutered and entire dogs (t-test, $p=0.89$) (Figure 4.10B). The addition of sex into the model did not result in the TRF to age regression statistically significant.

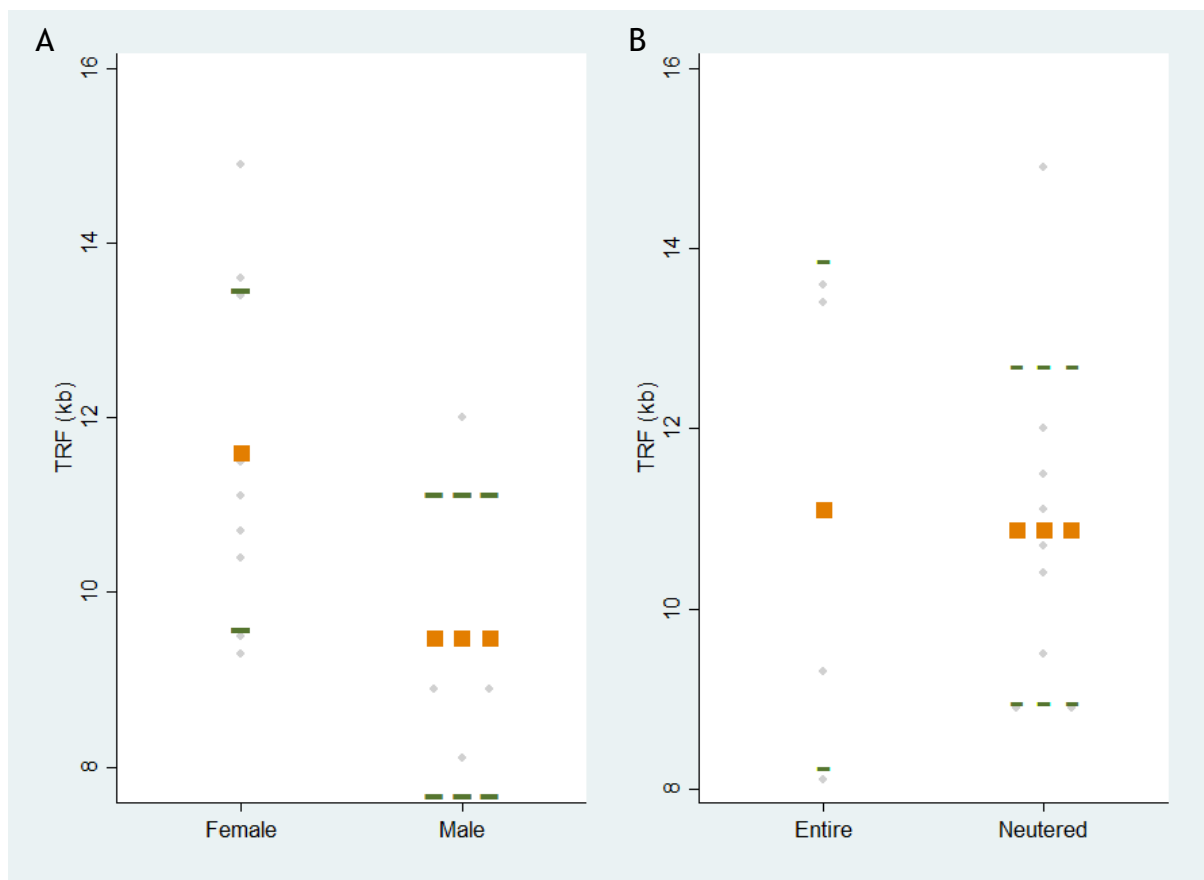


Figure 4.10 Leukocyte TRF by sex and neuter status

Dot plots of leukocyte TRF length by sex (A) and by if the dog was neutered (B). The orange squares represent the mean value and green dashes the 95% confidence intervals. Males had significantly shorter TRF lengths ($p=0.04$), but TRF length was not significantly different based on neutering status ($p=0.89$). $N=13$

Another factor on which information was available was the clinical diagnosis of the dogs, as they had all been seen at the Small Animal Hospital. The dogs were then divided into two groups: those with a malignancy diagnosed and those without. 8

dogs had a malignancy diagnosed, 5 did not. The dogs with a malignancy diagnosed had statistically shorter TRF lengths (t-test, $p=0.01$, Figure 4.11), with the malignancy-diagnosed dogs having a mean TRF of 9.75kb and those without 12.86kb.

Given that a diagnosis of malignancy seemed to have a significant association with telomere length, it was decided that samples from dogs with cancer should be avoided from this point forward. However, this is difficult to rule out absolutely for all these samples in practice, given that surplus blood was obtained from a referral centre where dogs are in a severe enough condition to warrant referral, and symptoms may be reflecting an undiagnosed cancer. In addition, cancer is common in dogs, with a median of 27% of mortality across breeds in the UK being due to cancer (Adams *et al.*, 2010).

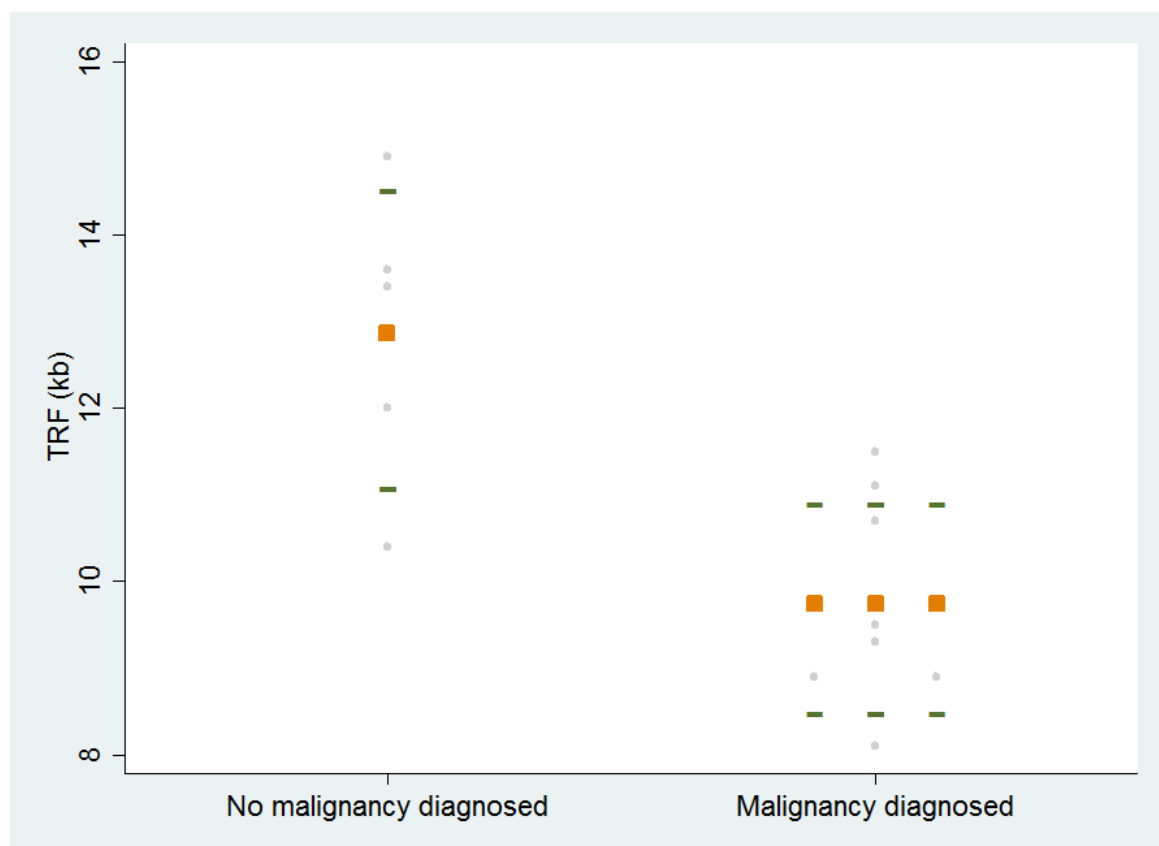


Figure 4.11 Leukocyte TRF by presence or absence of malignancy diagnosis

Leukocyte TRF by whether the dog had a diagnosed malignancy. There was a significant difference between the groups ($p=0.01$). $N=13$

4.3.5 Labrador retriever buccal TRF analysis

10 Labrador retriever buccal DNA samples were used for TRF analysis. These 10 dogs comprised of: 1 entire male, 2 neutered males, 5 entire females and 2 neutered females. Their ages ranged from 0.1 to 11 years, with a median age of 0.85 years. The dogs were attending a first opinion practice when the samples were collected and none of the dogs had major health problems. TRF length ranged from 3.8kb to 15.6kb. A Shapiro-Wilk normality test demonstrated that the TRF length data were non-normally distributed ($p=0.009$). The TRF lengths were therefore log transformed for analysis.

A linear model of age to log buccal TRF was not statistically significant (Figure 4.12, $F=0.43_{(1,8)}$, $R^2=0.05$, $p=0.53$). T-tests showed that there was no significant difference between TRF lengths in male and female dogs ($p=0.33$) and no significant difference between TRF lengths in neutered and entire dogs ($p=0.80$). However, when both age and neuter status were used as explanatory variables for the TRF length, the model was statistically significant ($F=16.05_{(2,7)}$, $R^2=0.82$, $p=0.002$). The relationship between age, neuter status and buccal TRF are illustrated in Figure 4.13.

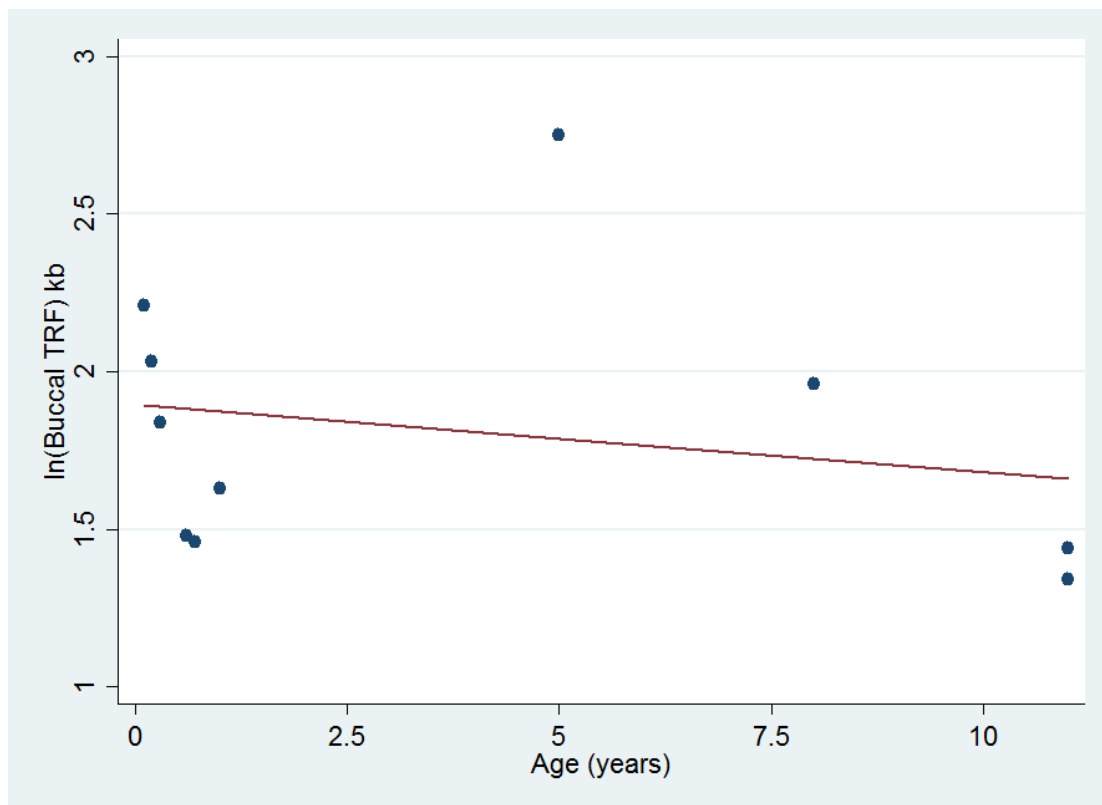


Figure 4.12 Buccal TRF length by dog age

Scatter plot of buccal TRF by age of a group of Labrador retrievers, with regression line. Linear regression was not statistically significant ($R^2=0.05$, $p=0.53$). $N=10$.

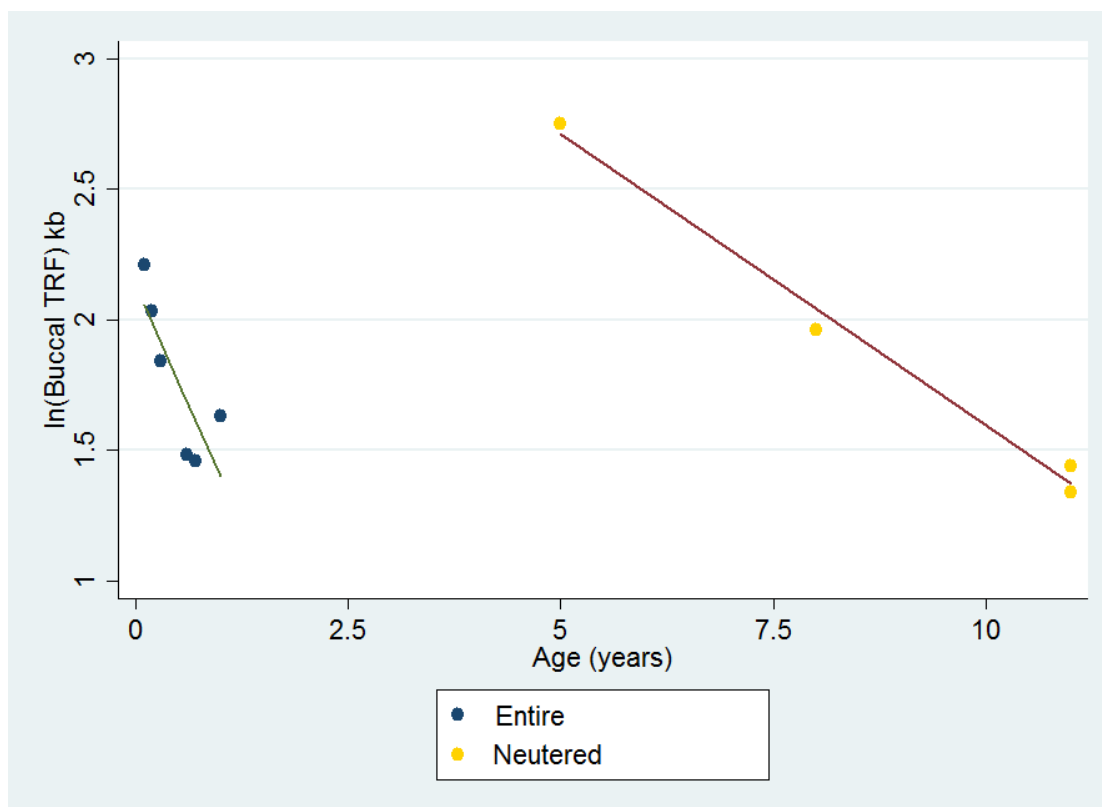


Figure 4.13 Buccal TRF by dog age and neuter status

Scatter plot of buccal TRF by age of a group of Labrador retrievers with regression line, with entire dogs and neutered dogs regressed separately.

4.3.6 qPCR optimisation samples

4.3.6.1 Leukocyte telomere length and demographic variables

To check the optimisation of the qPCR protocol, and to explore whether age-dependent attrition could be elicited using the RTL method, DNA was extracted from blood samples from 83 individual dogs and leukocyte relative telomere length was measured using qPCR. Only male dogs were used due to the potential effect of sex on telomere length seen in the TRF analysis experiments. 31 were castrated and 52 were entire. Samples were used from dogs of 16 different breeds, the information regarding the breeds is presented in Table 4-2.

Table 4-2 Dog demographics in qPCR optimisation sample group

Breed	Number of samples	Median age in years	Median weight (kg)
Akita	3	4.42	41.8
Bichon Frise	6	7.96	9
Border collie	5	4.71	21.3
Cavalier King Charles spaniel	7	7.45	11
Chihuahua	5	4.02	4.2
Dalmatian	7	6.89	29.5
Dobermann	3	7.58	44
Dogue de Bordeaux	4	5.54	47.5
English springer spaniel	9	5.43	19.7
Flat coated retriever	3	4.93	36
Greyhound	6	9.32	32.25
Miniature schnauzer	3	2.53	9.6
Rhodesian ridgeback	3	3.96	47.3
Siberian husky	3	8.44	28.6
Weimaraner	7	9.09	35.5
West Highland white terrier	9	6.53	8.5
All breeds	83	6.89	23.5

Relative telomere length (RTL) data were checked for normality using a Shapiro-Wilk test, with a resulting p value of 0.03. The data were therefore log transformed and a subsequent Shapiro-Wilk test gave a p value of 0.32. RTL values which were greater or less than two standard deviations away from the mean were excluded. This only had an impact upon miniature schnauzer data, and all RTLs from miniature schnauzers were excluded from further analysis due to the RTL measurements being very high.

Age of dogs ranged from 0.42 to 13.18 years. As shown in Figure 4.14, there was a significant negative correlation between relative LTL and age; however, there was a low R^2 value indicating wide variability in the data ($F=5.56_{(1,79)}$, $R^2=0.07$, $p=0.02$). Only 7% of the variation in RTL was explained by age. Therefore, further factors were investigated to explore whether more of the variation in RTL could be explained in the group of samples.

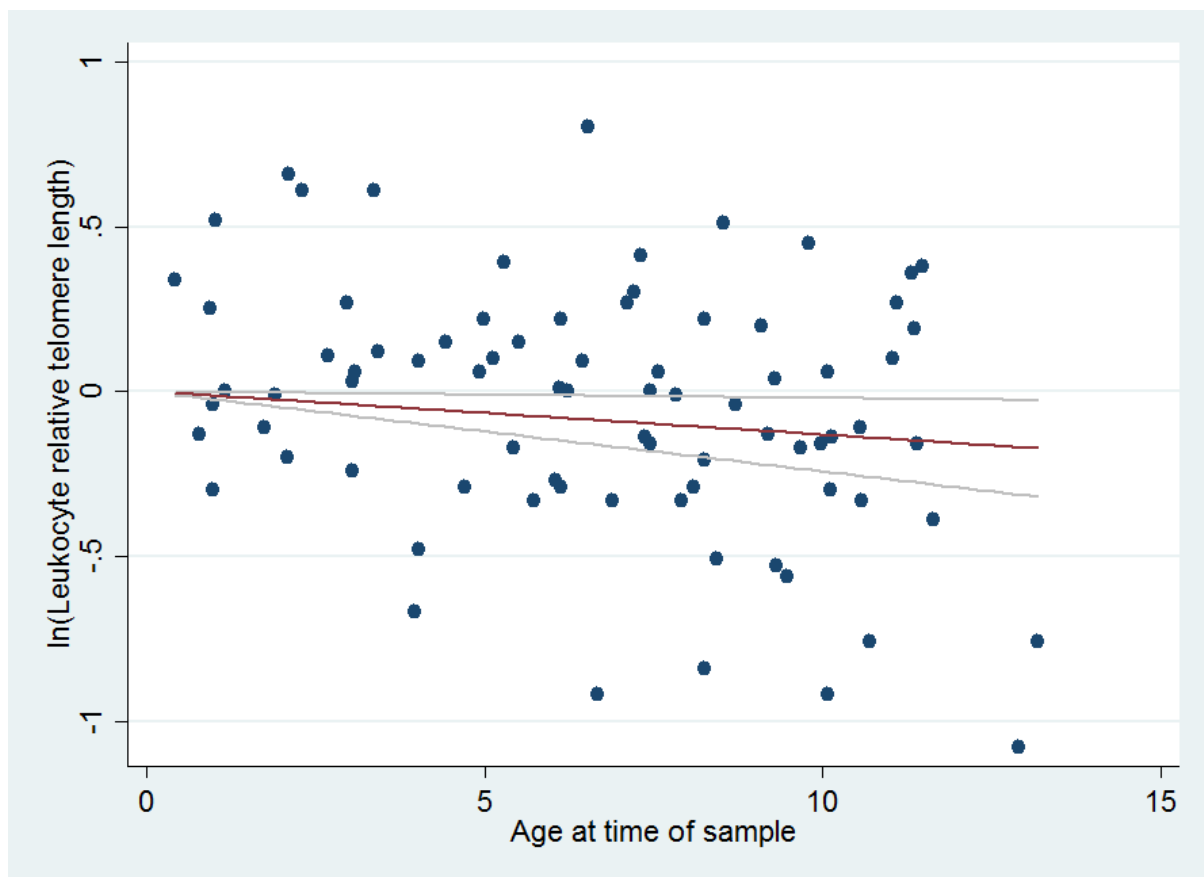


Figure 4.14 Leukocyte telomere length by dog age

Scatter plot displaying the negative correlation of natural log of relative telomere length in leukocytes by the patient's age in years, with regression line and 95% confidence levels ($R^2=0.07$, $p=0.02$). $N=80$.

The mean and standard deviation of RTL for each breed is shown in figure 4.15. There was not a significant relationship between breed and RTL ($F=1.53_{(15, 65)}$, $R^2=0.26$, $p=0.12$); however, when age was included as an explanatory variable the regression was significant ($F=2.62_{(16, 64)}$, $R^2=0.40$, $p=0.003$). In addition, the

number of dogs per breed was very small, limiting the ability to find significant differences.

Further analyses investigating potential sources of inter-individual variability in LTL in these samples are in Appendix 8.

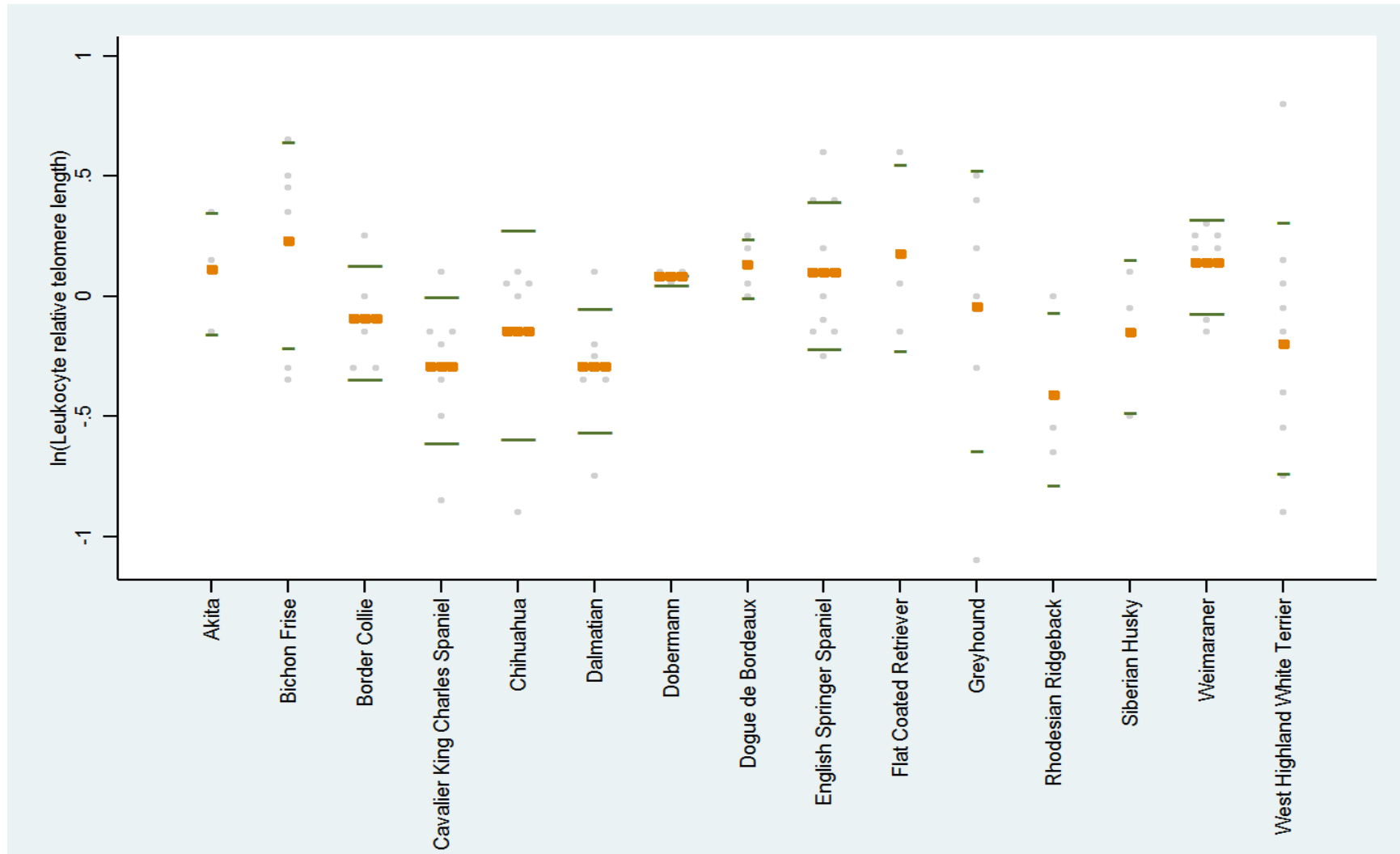


Figure 4.15 Leukocyte telomere length by breed

Dot plot of relative leucocyte telomere length by breed in 80 individual dogs. Mean value of LTL for each breed is displayed in orange, mean \pm standard deviation is displayed in green, with the individual values in grey. There was no significant difference in LTL between breeds ($p=0.12$)

4.3.6.2 Correlation between TRF analysis and qPCR

Fourteen of the blood samples used for qPCR RTL measurement were also used for TRF analysis to check the concordance of results generated using the two methods. As TRF analysis is considered the gold standard methodology for telomere length measurement, it was important to check that data generated by qPCR were concordant with that from TRF analysis. An 0.8% agarose gel was used. Figure 4.16A shows that there was a significant linear regression in matched sample telomere length analyses ($F=7.87_{(1,12)}$, $R^2=0.39$, $p=0.02$). Both linear regression and quadratic regression were performed, due to Aviv *et al* (2011) previously finding that quadratic regression between the two methods resulted in a higher R^2 value. The quadratic regression between the two methods in this study had a slightly lower R^2 value than the linear regression ($F_{(1,12)}=7.50$, $R^2=0.38$, $p=0.02$), Figure 4.16B. The Southern blot procedure was performed with a BSc Hons Veterinary Bioscience student under supervision. Data analyses of these blots shown were all generated by the author alone.

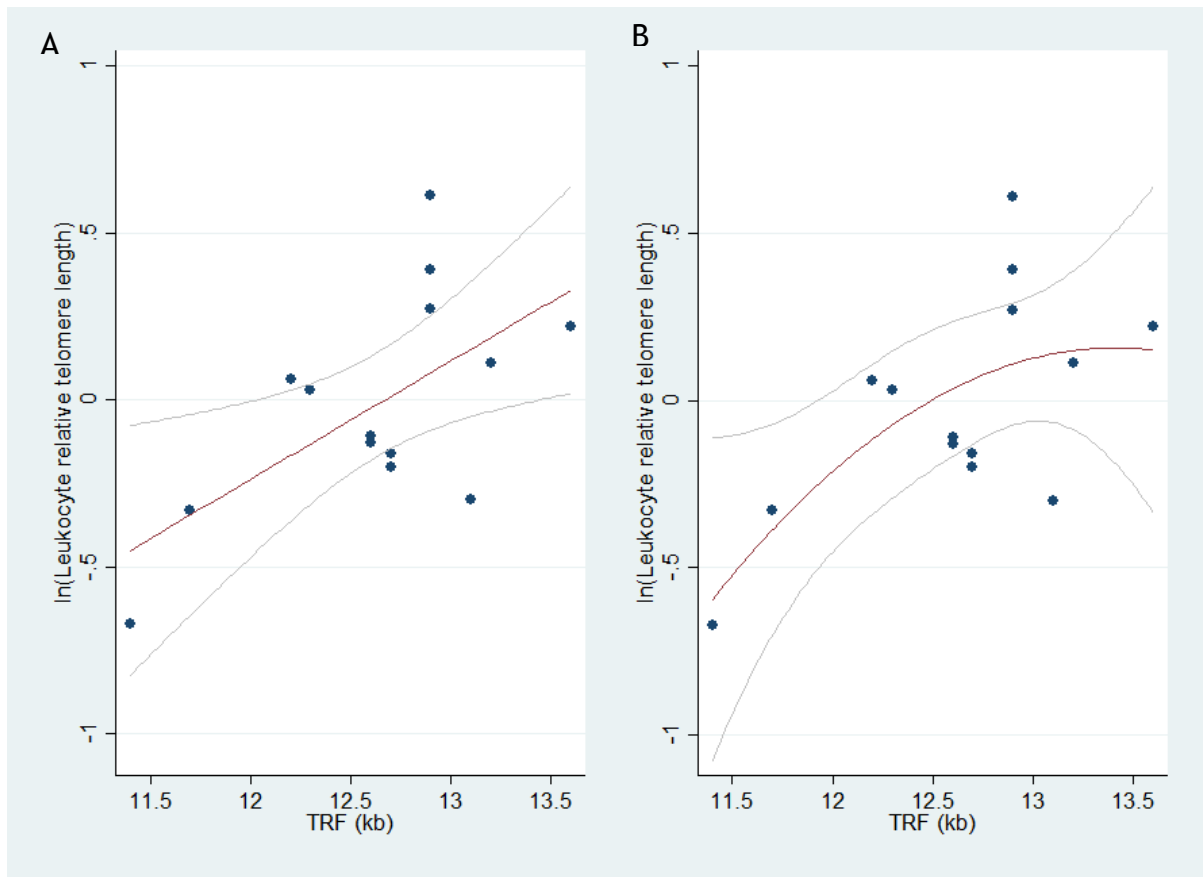


Figure 4.16 Paired analyses: qPCR and TRF measurements in the same samples

Scatter plot of paired telomere length measures in leukocytes, by qPCR RTL and TRF analysis, with regression line and 95% confidence intervals. Both linear regression (A) and quadratic regression (B) were statistically significant ($p=0.02$ for both). $N=14$.

4.3.6.3 Inter-assay variation

Southern blot TRF analysis

Control DNA samples (provided in the TeloTAGGG® kit) were run on each TRF analysis gel to enable comparison between gel measurements. The inter assay % CV was much higher when a 0.8% gel was used compared to a 0.5% gel (which was used in subsequent experiments in our laboratory), 8.9% versus 2.2%, respectively. Kit control TRF measurements were also shorter in the 0.8% gels, mean measurement 4.5kb, than the 0.5% gels, in which mean measurement was 7.2kb.

qPCR

Intra assay %CV was calculated for the sample triplicate repeats. Across the experiments in this chapter, the mean %CV intra assay for the telomere plates was 1.75% and 0.38% for the HBD plates. Two inter assay control samples were run on each plate. The inter assay %CV for RTL measurements of the sample made up of 20 blood samples was 3.64%. The inter assay %CV for MDCK RTL was far higher, at 19.64%.

4.3.7 Matched blood and saliva samples

The next set of experiments sought to investigate whether saliva telomere length would correlate with age, and if the saliva telomere length would correlate with LTL from the same animal. 24 pairs of matched leukocyte and saliva DNA from Labrador retrievers attending the Royal Dick School of Veterinary Studies Hospital for Small Animals were kindly provided by Dr Dylan Clements. The samples had already been DNA extracted in the laboratory of Professor Kim Summers, and 150ng of DNA from each sample was provided.

The dogs' ages ranged from 0.6 years to 11.25 years, with a median age of 4.45 years. There were 2 entire females, 7 neutered females, 3 entire males and 12 neutered males. Relative telomere length was measured by qPCR in all samples. One LTL result was excluded due to the result being greater than 2 standard deviations from the mean and another leukocyte RTL was excluded due to high intra assay variation. Saliva RTLs were significantly longer than leukocyte RTLs (paired t test, $p < 0.001$). Mean saliva RTL was 2.80, compared to 1.33 mean leukocyte RTL. Following Shapiro Wilk testing of the RTL values, no transformations of the data were required.

There was a strong negative correlation between age and LTL ($F = 21.15_{(1,20)}$, $R^2 = 0.51$, $p < 0.001$, Figure 4.17). There was also a significant negative correlation

between saliva RTL and age ($F=6.03_{(1,22)}$, $R^2=0.22$, $p=0.02$, Figure 4.18); however, the relationship was not as strong as that between leukocyte RTL and age.

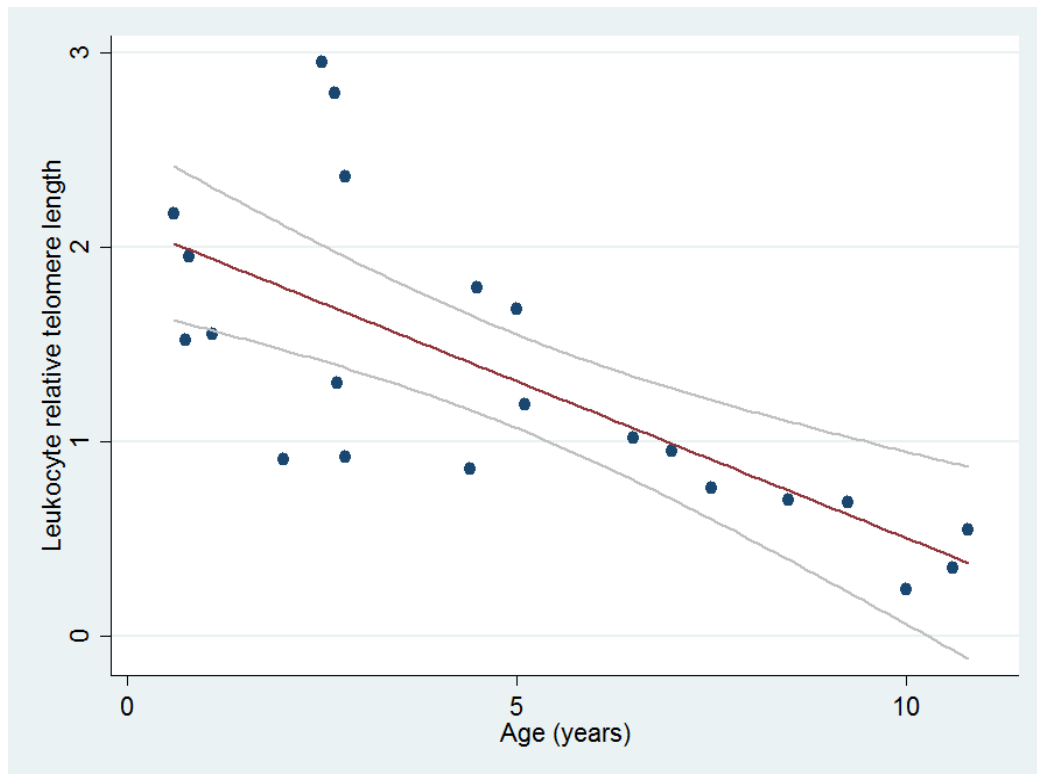


Figure 4.17 Leukocyte telomere length by age

Scatter plot displaying the negative correlation of relative telomere length in leukocytes by age, with regression line and 95% confidence intervals ($R^2=0.51$, $p<0.001$). $N=22$.

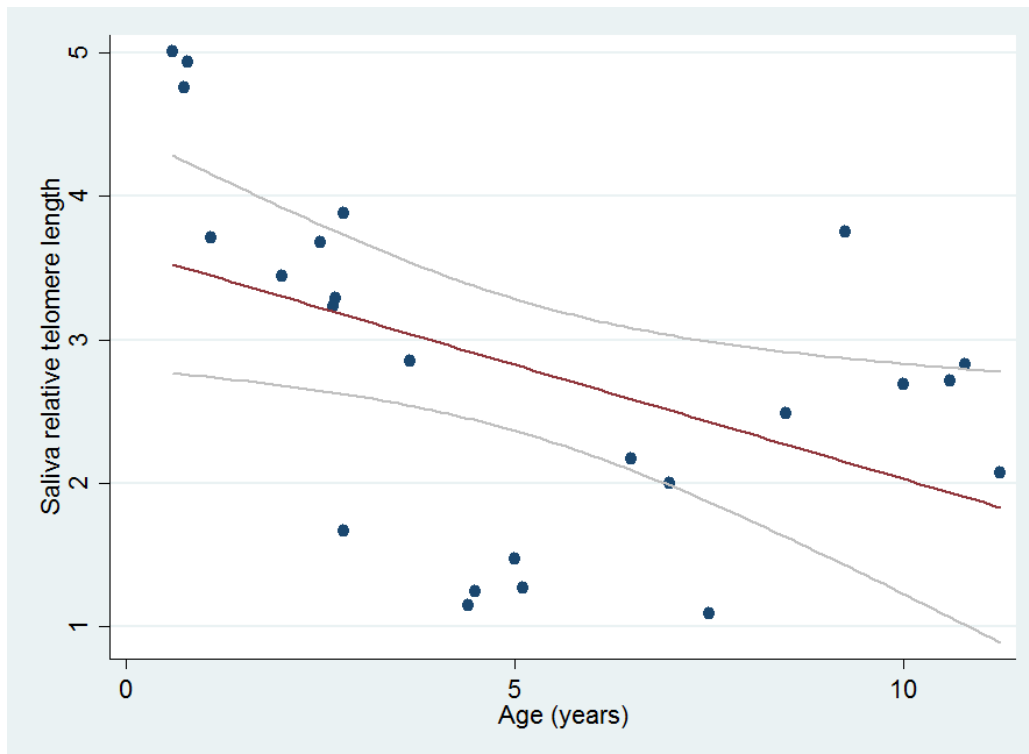


Figure 4.18 Saliva telomere length and age

Scatter plot displaying the correlation of relative telomere length in saliva by age, with regression line and 95% confidence intervals ($R^2=0.22$, $p=0.02$). $N=24$.

The mean leukocyte RTL value in females was 1.13 and 1.46 in males. This difference was not statistically significant (t-test, $p=0.36$). The mean saliva RTL in females was longer, at 3.01, compared to 2.63 in males. Although, the difference was again not statistically significant (t-test, $p=0.46$). In addition, there was no significant difference between neutered and entire dogs in leukocyte RTL (t-test, $p=0.73$) or saliva RTL (t-test, $p=0.50$). Grouping the dogs into four sex categories: female entire, female neutered, male entire and male neutered, there was a significant difference in saliva RTL between the groups (one-way ANOVA, $p=0.04$). As can be seen in Figure 4.19, entire males had shorter saliva RTLs than the other sex groups. There was not a significant difference seen in leukocyte RTL between the four sex groups (one-way ANOVA, $p=0.85$, Figure 4.20). The addition of this variable strengthened the association between saliva RTL and age ($F=4.65_{(4,19)}$, $R^2=0.49$, $p=0.009$).

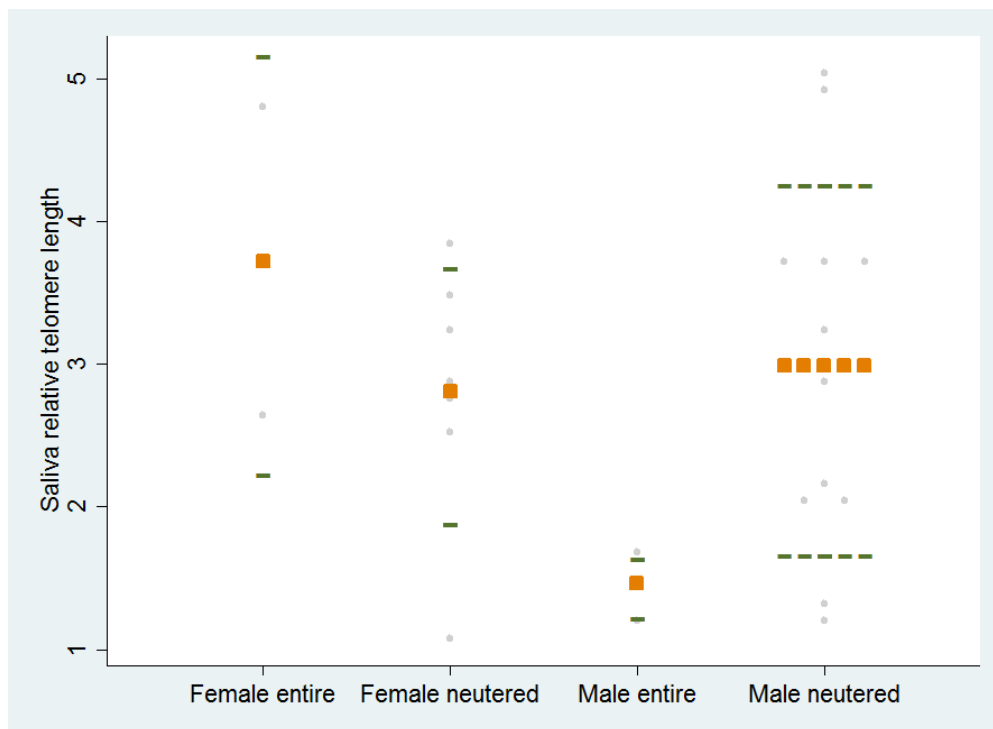


Figure 4.19 Saliva telomere length by sex and neuter status

Saliva relative telomere length by sex and neutering status of the dog. There was a significant difference between the groups (one-way ANOVA, $p=0.04$)

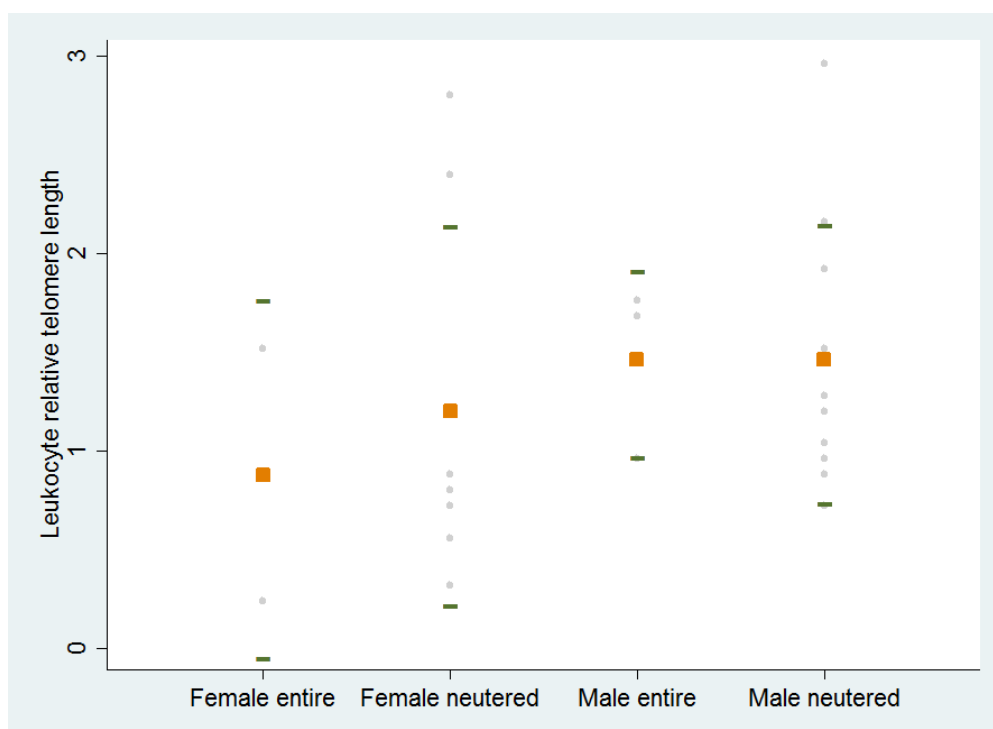


Figure 4.20 Leukocyte telomere length by sex and neuter status

Leukocyte relative telomere length by sex and neutering status of the dog. There was no significant difference between the groups ($p=0.85$)

There was a positive trend between leukocyte and saliva RTL paired measurements, however this relationship was not statistically significant ($F=4.123_{(1,20)}$, $R^2=0.17$, $p=0.06$, Figure 4.21). However, if the 4 group sex variable was added as an explanatory variable, the relationship was statistically significant ($F=3.82_{(4,17)}$, $R^2=0.47$, $p=0.02$). Figure 4.22 displays a Bland Altman plot for measurements using the two tissue types, plotting the mean relative telomere length and the difference between the two values. There was a significant positive correlation between the mean and difference, such that the longer the mean relative telomere length, the larger the difference between the leukocyte and saliva RTL values ($R^2=0.24$, $p=0.02$). However, no measures fell outside the limits of agreement (shown by the 95% confidence interval of the mean of difference lines in Figure 4.22).

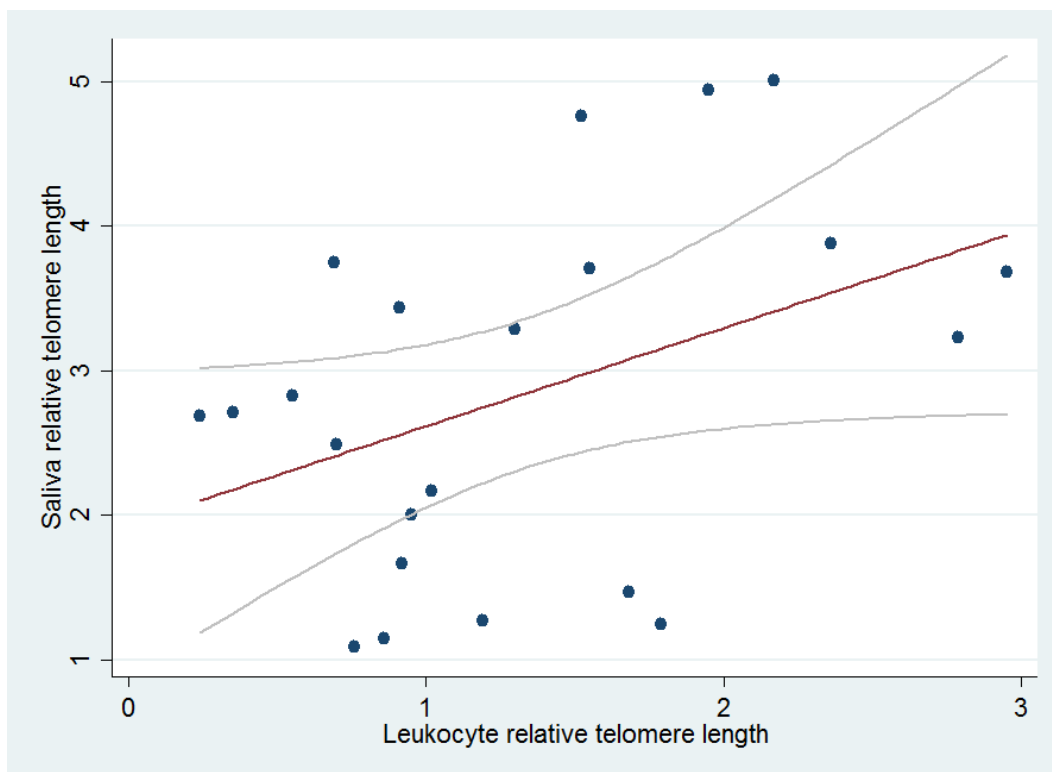


Figure 4.21 Saliva and leukocyte telomere lengths within individual dogs

Scatter plot displaying the correlation of relative telomere length in saliva by the relative leukocyte telomere length, with regression line and 95% confidence intervals ($R^2=0.17$, $p=0.06$)

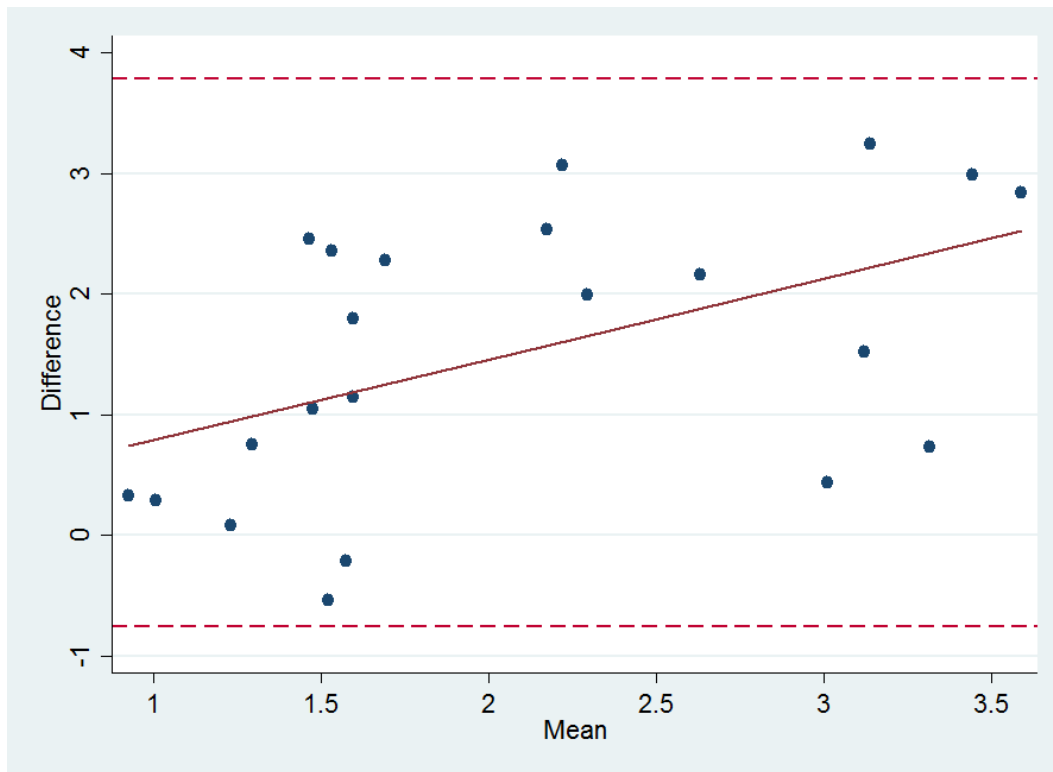


Figure 4.22 Bland Altman plot

Bland Altman plot of saliva and leukocyte relative telomere length paired measurements. The dashed lines represent the limits of agreement.

4.3.8 Leukocyte DNA samples from Manchester

50 leukocyte DNA samples from Labrador retrievers were kindly provided by the University of Manchester. The intention of these was to complement the data generated from the Edinburgh samples. The samples were DNA extracted in Manchester. Three samples provided had insufficient DNA quantity to be included. qPCR analysis was performed on the remaining 47 samples. Two samples were then excluded, as one failed to amplify on both the telomere and reference gene plates and another had an intra-assay variation higher than the accepted level.

Following Shapiro Wilk testing, the data were not transformed. There was no significant correlation between RTL and age and, in fact, a positive trend was observed between the two variables ($F=3.387_{(1,43)}$, $R^2=0.07$, $p=0.07$, Figure 4.23). There was no significant difference in RTL between males and females (t-test,

$p=0.75$), neutered and entire dogs (t -test, $p=0.71$) or in the four sex groups (one-way ANOVA, $p=0.35$, Figure 4.24).

To investigate the difference in results in these data compared to previous results, the remaining DNA from the samples was quality checked using spectrophotometry. The mean 260/280 ratio was 1.82 and ranged from 1.66 to 1.99, and so several samples met the required 1.8 cut off value for quality. However, the mean 260/230 value was 0.38 and ranged from 0.30 to 0.52, well below the accepted level and indicative of the presence of potentially inhibitory substances. Therefore, these results were not added to the previous data, and served as a warning as to the importance of DNA samples meeting quality requirements if they are to be used in the qPCR assay. The HBD C_{Ts} for the samples correlated negatively with the 260/230 ratios ($p=0.02$), all other correlations were not statistically significant.

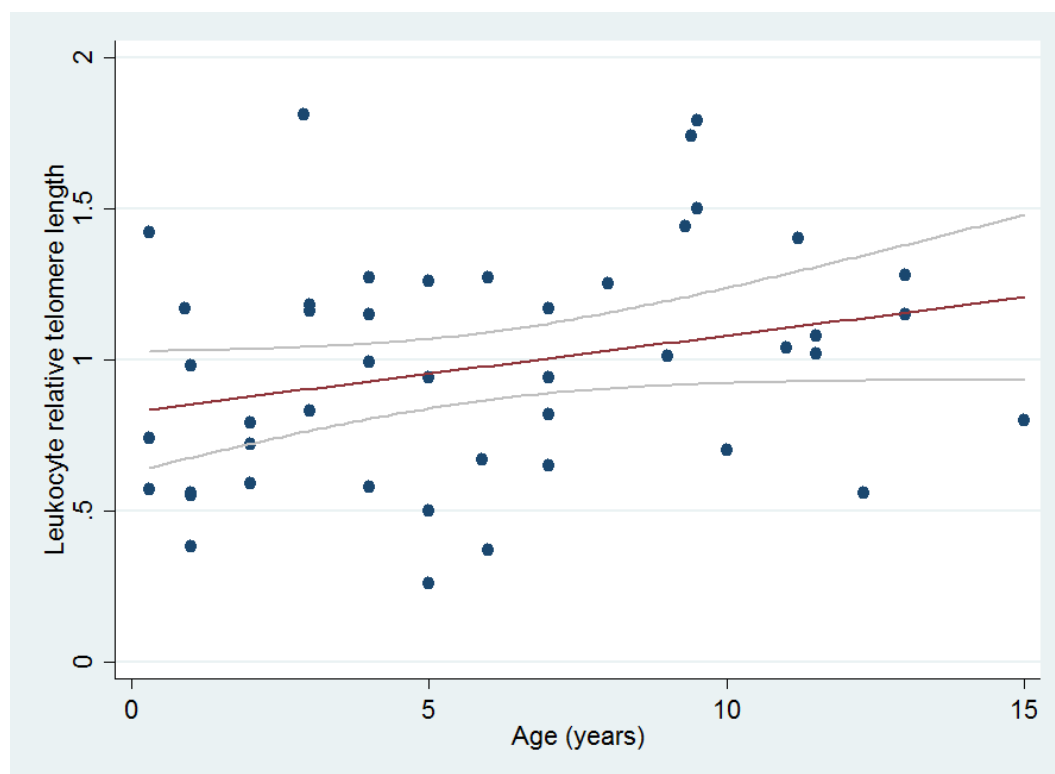


Figure 4.23 Leukocyte telomere length by age in dog samples collected in Manchester with regression line and 95% confidence intervals ($R^2=0.07$, $p=0.07$). $N=47$.

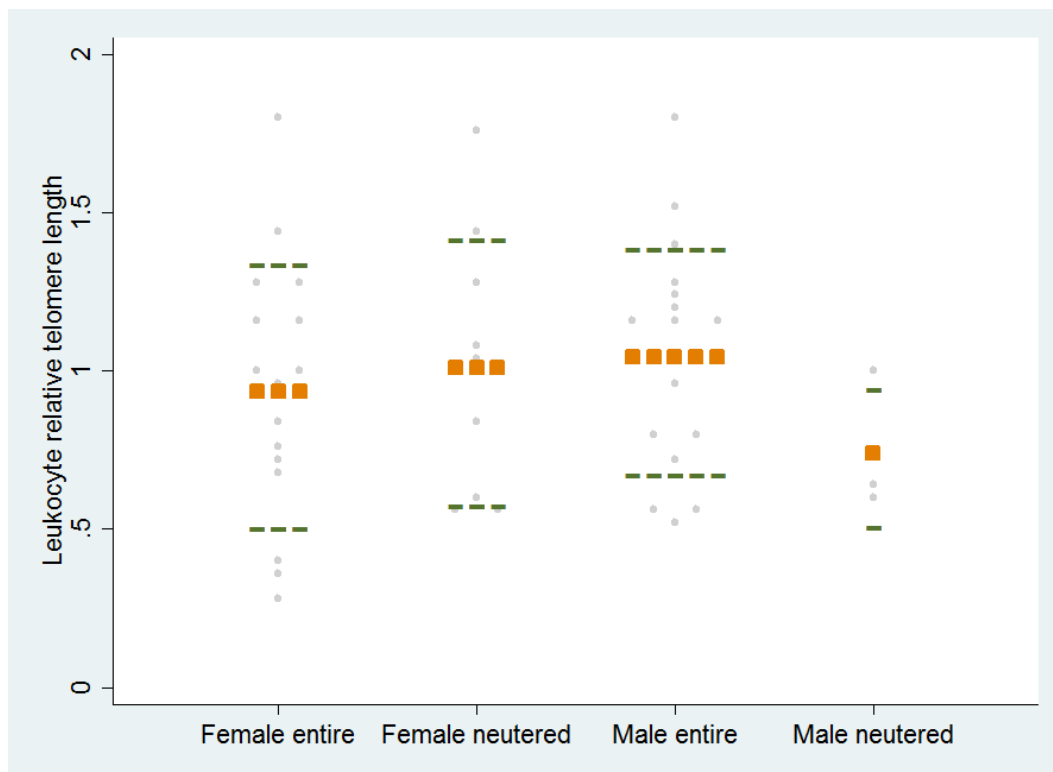


Figure 4.24 Leukocyte relative telomere length by sex status of the dog in samples from Manchester.

There was no significant difference in leukocyte RTL between the sex groups ($p=0.35$). $N=47$.

4.4 Discussion

4.4.1 Telomere length measurement methodology critique

Work described in this chapter aimed to establish a reliable method for measuring telomere length in canine clinical samples. The absolute telomere length qPCR method, as first described by O'Callaghan *et al.* (2008), was decided against after results generated were found to be highly variable and approximately 30 times longer than that measured in humans. Others have found issues with this method, for example Wong *et al.* (2014) decided not to use the method because of the effect of variability while working with picograms of oligomer standard affecting the end results. This may have been part of the reason for variability between runs found here.

While it may be beneficial to have an absolute, as opposed to, relative telomere length to enable comparability between laboratories and studies, it was unclear from the existing literature as to what lengths should be expected in the canine samples, and therefore the relative telomere length qPCR was used moving forward. Other studies have converted RTL data to length in kilobases by measuring samples by both TRF analysis and RTL qPCR, plotting the resulting data and using the resulting equation of the regression line to convert the RTL data to TRF in kb (for example Ehrlenbach *et al.*, 2009; Needham *et al.*, 2013). This would be a potential option to be used for canine samples in the future.

Multiplexing the RTL qPCR assay has been employed in other studies, using the method as described by Cawthon (2009). By using a multiplexing approach, both the telomere and reference gene PCRs are performed in the same well, so that only one plate is required to be run per RTL assay, saving time and money. This also has the advantage of reduction in variation due to different amounts being pipetted on each plate. Cawthon found that this method improved the correlation between RTL and TRF measurements compared to the original singleplex method. This method was not employed here as the aTL method requires singleplex qPCR and the primers, annealing temperature, and so forth were optimised with the aTL method in mind. However, this could be something to consider in the future. It

has been successfully employed in cattle samples (Brown *et al.*, 2012) as well as human samples (e.g. Shen *et al.* 2011).

One issue with telomere qPCR is the effect of well position on RTL. One study found well position accounted for 10.4% of the variation in RTL values, and a method to improve the assay in this study could be to employ a well position correction as performed by Eisenberg *et al* (2015). The variation may be due to limitation in uniformity in temperature across the thermal block in the qPCR machine (Javorschi-Miller and Delgado Orlic, 2011). Machines such as Rotor-Gene (Qiagen) have much lower variation in temperature but were not available for use in this study.

A potential alternative to qPCR for the future of telomere length measurement is the use of digital PCR (dPCR). dPCR uses an “all or none” approach (Sykes *et al.*, 1992). The test sample is partitioned into a large number of reaction chambers, which are then determined to be positive or negative based on the presence or absence of the target of interest after amplification (Baker, 2012). The proportion of positive partitions is then used for quantification calculations (Huggett *et al.*, 2013). Digital PCR has advantages over traditional PCR including being more sensitive and having a higher reproducibility (Baker, 2012). Higher sensitivity may be useful for detecting small changes in telomere length, for example changes due to cigarette smoke exposure as will be examined in forthcoming chapters. Use of dPCR has limitations for repeats of the same sequence (Huggett *et al.*, 2013) such as occurs in telomeres. For the application of dPCR in telomere length measurement, extra preparation steps may be required, potentially separating the telomere from the rest of the chromosome and there may be other issues encountered such as the long length of the telomere. However, the author notes a “digital assay for telomere length” (Litterst and Ugozzoli, 2016) patent has been submitted, suggesting that this technology may be used for telomere length measurement in the not-too-distant future. No published studies employing dPCR

for telomere length could be found, however a digital PCR based method for telomerase quantification has been published (A. T. Ludlow *et al.*, 2014).

There was a significant linear regression between qPCR and TRF measurements in this chapter. Unlike in Aviv *et al* (2011), the R^2 value was not improved by fitting a curvilinear model to the data. However, the polynomial regression was significant. This could be due to the use of a 0.8% agarose gel for TRF analysis in this chapter, resulting in underestimation of TRF length in some samples. In data not included in this thesis, later experiments were performed using a 0.5% gel for TRF analysis. The control sample was found to have a longer TRF and interassay variation was reduced. The main reason for using TRF analysis, however, was to show the effectiveness of the qPCR method, and a significant linear regression was displayed. Steps could be taken in the future to further optimise the TRF protocol, to see whether the regression between the two methods changes, and provide a means of converting RTLs to TRF length as described earlier in the discussion.

Examining the TRF analysis method paper by Kimura *et al* (2010), they recommend a 0.3% agarose gel for when TRF lengths of 16-17kb are expected, so a further reduction in gel percentage could be used here. Although, of course this would make the gel very delicate and it would require very gentle handling. 0.3% gels were used to examine TRF lengths in tissues from CAST/Ei mice, a wild derived strain with relatively short telomeres for mice, and the mean lengths in different tissues varied between 10.3 and 17.6kb (Cattan *et al.*, 2008). These lengths are similar to those in dog tissues found by Benetos *et al* (2011), but on the whole, shorter than those found in dog tissues by Cadile *et al* (2007) and Nasir *et al* (2001).

A further option to improve the resolution of longer telomere fragments, is to use a non-conventional form of gel electrophoresis, namely pulsed field gel electrophoresis (PFGE). PFGE can separate DNA fragments up to approximately

10mb in size, compared to roughly 50kb in conventional gel electrophoresis (Herschleb, Ananiev and Schwartz, 2007). PFGE was developed by Schwartz & Cantor (1984) and uses “non-uniform, alternately pulsed, electrical fields”. One difficulty with PFGE is the requirement of specialist equipment. There are multiple types of PFGE. One version is called field-inversion gel electrophoresis (FIGE), which has the advantage of requiring minimal specialist equipment (Carle, 1992). FIGE was used to measure pig tissue TRFs, which were shown to range from 9 to over 50kb (Fradiani *et al.*, 2004).

One issue in this chapter which prevented the identification of long telomere fragments, aside from the gel percentage and electrophoresis technique, was the use of the molecular weight marker provided in the TeloTAGGG kit, with the largest fragment being 21.2kb. A marker with fragments up to 48.5kb is commercially available and would be preferable for future experiments. Two method variations have been used in publications to improve the precision of the TRF analysis protocol. One is to strip and reprobe the blot with a loading control to correct for differences in amounts of DNA loaded into each well. Schmitt *et al* (1994) used a minisatellite (CAC)₅ probe for this purpose. The other is to resolve a molecular weight marker in the sample lanes with the samples to correct for different rates of DNA migration across the different regions of the gel. The telomere probe is used first, the membrane is stripped and then a molecular weight marker is used (Vasan *et al.*, 2008). However, while these techniques may improve precision, they are not written about in many published studies using TRF analysis, and the key issue in this study appears to be insufficient resolution of large telomere fragments in the canine samples.

The interassay variation in the qPCR assay was very different depending on the sample used to calculate this value: blood or MDCK. In addition, the MDCK sample was shown to be an inappropriate reference sample, so it would be better to have two blood control samples in future studies. Cell lines have been used as control samples in telomere studies previously (e.g. Fehrer *et al.* 2006), but here it was

decided against due to the high RTL value and variability. Interassay variation in RTL measurement in published studies varies greatly, and is not always reported. Published examples of RTL interassay %CV include: 3.17% (Ferlin *et al.*, 2013), 4.9% (Aydinonat *et al.*, 2014), 5.8% (Elbers *et al.*, 2014), 6.45% (Aviv *et al.*, 2011), 8.1% (Ahola *et al.*, 2012), 12.04% (Heidinger *et al.*, 2012) and 28% (Shen *et al.*, 2007). 3.64% interassay variation is therefore at the lower end of the range found in the literature. Interassay %CV is generally lower in TRF analyses than qPCR, for example 1.5% (Elbers *et al.*, 2014), 1.7% (Fitzpatrick *et al.*, 2011), 1.74% (Aviv *et al.*, 2009), 3.6% (Kadi *et al.*, 2008) and 3.7% (Strandberg *et al.*, 2012). The interassay variability of TRF analysis in this chapter, 8.9%, is clearly higher than those reported in current literature.

The RTL in the qPCR optimisation samples were significantly different depending on the DNA extraction kit used. Differences in RTL based on DNA extraction method were previously found by Cunningham *et al.* (2013). They compared RTL in samples using phenol chloroform, Puregene (salting out method) and Qiagen column extractions, and found Qiagen extracted samples had significantly shorter RTLs than when the other two methods were used. In contrast, when Denham *et al.* (2014) compared RTLs in samples DNA extracted by Qiagen columns, Purelink kit (salting out method) and a high salt method by Lahiri and Numberger (1991), they found RTLs measured in samples DNA extracted using Qiagen columns to be significantly longer than those extracted by the high salt method. RTLs in samples extracted using Qiagen and Purelink kits were found to be not significantly different. Tolios *et al.* (2015) compared a bead based extraction, column extractions (using Qiagen, Macherey Nagel and 5prime columns), Stratec precipitation-based kit and an isopropanol precipitation protocol. When a singleplex qPCR protocol was followed, and samples had not been frozen, they found the Macherey Nagel extracted samples had significantly shorter RTLs than those extracted using the Qiagen kit. However, if the samples had been frozen, there was no significant difference in RTL measurements. Other differences were found in RTL of samples extracted by the different methods, notably the isopropanol precipitated samples had shorter RTLs compared to other extraction

methods in multiple pair wise comparisons. Cunningham *et al* state the reason for shorter RTL in Qiagen extracted samples could be the shearing of DNA through vortexing as part of the protocol, multiple wash steps and only the DNA that has remained on the column will be collected in the resulting elute. In addition, they compared RTLs to TRF analysis measurements and found that the Qiagen extracted samples still had shorter telomere lengths. They also performed a literature review, and suggest that from their findings, use of Qiagen columns may result in more false negative results in the link between short telomere length and cancer risk, compared to using phenol chloroform or salting out DNA extraction methods. 260/280 ratios have been found to be lower, however, in canine blood samples extracted using phenol chloroform, compared to Qiagen columns (Clements *et al.*, 2008). Puregene extracted DNA samples were found to have higher 260//280 ratios than Qiagen extracted samples in the same study. The phenol chloroform extracted samples showed significantly higher PCR inhibition than the other samples. Denham *et al* (2014) found that Qiagen columns produced DNA with significantly higher 260/280 ratios than Purelink-extracted DNA. In addition, they found significant correlations between both 260/280 and 260/230 ratios with RTL. In this chapter, correlations were found between 260/280 ratio and telomere C_T in one data set, and 260/230 and HBD C_T in the Manchester data set. The Manchester samples had 260/230 ratios which fell well below the purity rules set for samples extracted by the author. This was suspected to be the reason that no significant correlation was found between RTL and age in this group. In addition, as the 260/230 ratios were lower when the buccal swabs were phenol-chloroform extracted. For future experiments, DNA was extracted from swabs using column extraction where telomere length was to be measured using qPCR.

An issue which may have had an impact upon results when using surplus samples from the VDS, is the stability of RTL with sample storage conditions. Zanet *et al* (2013) found that when EDTA anticoagulated blood samples were stored at 4°C for more than 4 days, there was an increase in RTL. The samples used here will have been stored at 4°C for at least a week, then frozen. Zanet *et al* also found that freeze-thawing samples did not affect RTL. Without further investigation, it

cannot be known the variability of the impact of the storage conditions of the samples used here, and if there are any factors which may affect the stability of the RTL. In this study, white cell count and differential were related to 260/230 ratio, as well as telomere C_T and HBD C_T . Age of dog also was associated with DNA quality ratios.

Baker III and Sprott (1988) comprised a list of criteria which biomarkers of ageing should fulfil. This list included that “biomarkers should change independently with the passage of time and reflect physiologic (functional) age”. The linear model of age to leukocyte TRF was found not to be significant, but there was a significant association with sex and presence or absence of malignant disease and TRF. The TRF analysis protocol could have been improved, which may have improved the relationship between age and TRF; however, differences in TRF between the other factors were observed. The linear regression between buccal TRF and age was highly significant when neutering status was included as explanatory variables, with the R^2 value reaching 0.82. The dogs from which the buccal swabs were collected had no major health problems and were attending a first opinion practice, whereas all other samples were from dogs attending referral centres. As was seen throughout the chapter, disease status, namely having cancer or not, had a significant association with telomere length, so having samples from dogs unwell enough to attend a hospital may have impacted the ability to identify the factors which would have a relationship with telomere length in a healthy dog population. The Edinburgh samples were from dogs attending an orthopaedic clinic, and the regression of RTL to age in these dogs was significant, with a R^2 of 0.51, possibly because some of the presenting problems were “elbow dysplasia” and “cruciate disease”, which may have had less of an association with RTL than a malignancy. Without further studies, it is not possible to know the relationship of each presenting problem with telomere length in dogs.

While the R^2 of the regression between RTL and age in the qPCR optimisation samples was only 0.07, this value is in line with those stated in other studies using

human and dog samples. Several studies examined did not state the R^2 value, but the following examples were found: R^2 of 0.026 and 0.075 in both sexes and male only, respectively, in pet dogs (Fick *et al.*, 2012); R^2 of 0.06 between leukocyte TRF and age in pet dogs (Benetos *et al.*, 2011); R^2 of 0.145 in samples from healthy humans and R^2 of 0.079 in humans with COPD (Houben *et al.*, 2009); and R^2 of 0.04 in humans with COPD and R^2 of 0.09 in humans with HIV (Liu *et al.*, 2015). Neither dog study stated whether the dogs had underlying health concerns or not.

4.4.2 Associations between characteristics of dogs and telomere length

Due to the number of cases used in this chapter, all cancer patients were grouped together. Other studies have found varying effects of cancer on LTL, depending on the type of cancer diagnosed. For example, Hou *et al* (2009) found patients with gastric cancer had significantly shorter LTL than controls, and Jang *et al* (2008) found lung cancer patients had significantly shorter LTL than a group of age, sex and smoking status matched controls. In contrast, Sun *et al* (2015) observed that individuals with endometrial cancer had longer leukocyte telomere length than controls. Shen *et al* (2007) found no significant difference in LTL between breast cancer patients and unaffected sister controls. It would be interesting to investigate if there is variability in telomere length with different cancer types in dogs.

The RTL in dogs in this chapter was found not to be statistically significantly associated with breed lifespan. The lack of correlation between LTL and lifespan is rather different to the result found by Fick *et al* (2012). They found a strong ($p < 0.0001$) regression between breed life expectancy and LTL. A possible reason for this difference is the study population used in this chapter; none of the dogs which were known to have died reached their breed life expectancy. A healthier population may be required to generate similar results seen in the Fick *et al* study. Fick *et al* (2012) also examined the likelihood of death due to disease in different

body systems and cancer mortality based on breed with LTL. Data from the UK Kennel Club were used here to compare percentage of deaths in each breed for the four most common causes of death in dogs: cancer, cardiac illnesses, old age and urological illnesses (no data were available for Chihuahuas or West Highland white terriers, and only limited data were available for Dogue de Bordeaux) to RTL and age adjusted RTL. No cause of death was significantly correlated to RTL, age adjusted or not (data not shown). In addition, using data from Fleming *et al* (2011), who examined cause of death in breeds in America (and was the study for which Fick *et al* (2012) obtained their mortality data), no significant correlations were found between RTL and the breed causes of death (data not shown).

The dogs that were known to have died soon after sampling had shorter RTL when age was added in as an explanatory variable. The number of dogs that died may have been underestimated if the dog's death was not communicated to the small animal hospital. Other studies have found a link between short telomere length and mortality. Epel *et al* (2009) showed that, in human males, telomere shortening rate was linked to increased cardiovascular mortality. Fitzpatrick *et al* (2011) found that persons in the shortest quintile of LTL were 60% more likely to die in the study follow up period than those in the longest quintile. However, in two studies of LTL in elderly persons, there was no significant association between LTL and subsequent mortality found (Bischoff *et al.*, 2006; Harris *et al.*, 2006).

The figures for breed lifespan used in this chapter were generated using breed median age of death from three studies (Michell, 1999; Adams *et al.*, 2010; O'Neill *et al.*, 2013). Michell sent questionnaires to owners using addresses from a pet insurance company and at Crufts Dog Show and asked about their "last dog". Adams *et al* sent questionnaires to breed clubs and asked about all dogs which had died in the past 10 years. O'Neill *et al* used data from primary veterinary practices. Each method had strengths and limitations. For example, use of breed clubs only meant that the sample was not random, but Adams *et al* was the only study of UK dogs where information on all the breeds used in this chapter was

available. O'Neill *et al* found there were issues with missing data. Questionnaire data could not be corroborated with the dogs' veterinary practices, so cause of death may not be entirely reliable. Breed age at death in all studies included data from both euthanised dogs and dogs which died naturally, as well as deaths due to trauma and behavioural issues. In fact, trauma was the most common cause of death in Chihuahuas, one of the breeds in this study, in an investigation of causes of death in American dogs (Fleming, Creevy and Promislow, 2011). To check whether there was an effect of the sample group or method, the breed lifespan from each of the studies was tested for correlation with RTL, both age adjusted and not. There was no significant correlation with the breed life expectancy and RTL using the Adams *et al*, O'Neill *et al* or Michell figures (data not shown).

Some studies have suggested that telomere length in early life is predictive of lifespan. For example, Fairlie *et al* (2015) found that female soay sheep with longer LTL in the first two years of life had improved survival rates, but there was no link in later life. RTL at 25 days of age was seen to be predictive of lifespan in Zebra finches (Heidinger *et al.*, 2012). Examining the youngest 10 dogs in the qPCR optimisation samples, there was, in fact, a significant negative correlation between RTL and breed life expectancy (Spearman's $\rho = -0.64$, $p = 0.047$).

Variables related to breed, other than lifespan, were associated with RTL when age was included as an explanatory variable. The estimated effective population size, a measure of inbreeding, was inversely related to age adjusted RTL, such that dog breeds with a higher rate of inbreeding had longer telomere lengths. One weakness of using the estimated effective population size to assess the level of inbreeding was that figures were not available for greyhound or Rhodesian ridgeback breeds due to there being increasing genetic diversity in these breeds. Although, interestingly, the average RTL for the Rhodesian ridgebacks was the shortest average RTL out of the breeds, both unadjusted and age-adjusted. Greyhounds had on average the eighth shortest RTL but, as will be discussed later, dogs with lower cephalic indices had longer RTLs. The finding that inbreeding was

associated with longer telomere lengths is in line with Manning *et al* (2002), who found that mice from inbred lines had longer LTL than outbred strains of mice. However, Leroy *et al* (2014) found inbreeding was associated with reduced survival to two years of age in 7 breeds of dog. Shorter RTL was associated with mortality in this chapter, yet inbreeding was associated with longer LTL.

The cephalic index was significantly related to RTL in a model with age included. Cephalic index (CI) is not routinely measured and recorded, so breed averages were used for CI values. The variability of the skull conformations within each breed was not known. Dogs with lower CIs had longer RTLs when age was included in the model. Higher CI has been associated with health problems and reduced lifespan, as discussed in section 1.8.3. However, thus far the RTL measurements have not reflected breed lifespan, so possibly the association between RTL and CI reflect the health of the individuals. There were no studies of RTL and cephalic index found to compare the results to.

Breed averages were also used for height, due to this information not routinely being measured and recorded. While this may have led to some inaccuracy, Sutter *et al* (2008) demonstrated low phenotypic variation within breeds, with at least 70 percent of dogs conforming to their breed's height standards. Therefore, they stated that Kennel Club (in their case American Kennel Club) measurements are a good proxy for actual height measurements. Weight was not significantly related to RTL, yet weight was significantly correlated with lifespan. Initially, there was no significant regression found between height and RTL, yet height was included in one of the final models, with an estimated coefficient of -0.12 indicating a negative relationship, such that shorter dogs had longer RTLs. The link between weight and lifespan was expected, given that this relationship has been found in multiple previous studies (e.g. O'Neill *et al*. 2013; Galis *et al*. 2007). Kraus *et al* (2013) examined breed mortality data, including age at death and the average weight for the breeds. They concluded that large dogs die at a younger age because they "age quickly". They found a positive link between body size and the

absolute speed at which mortality hazard increased. However, they found no significant relationship between size and age at which a mathematically calculated onset of senescence occurred Benetos et al (2011) speculated that the variation in the difference between leukocyte and skeletal muscle telomere length was due to the longer growth period of larger dogs. Examining the RTL of dogs under two years of age in this chapter, there was still no relationship with weight. While Fick et al (2012) did not comment on correlation between LTL and weight, it can be seen in their study that Labrador retrievers, a large breed, had the longest average age-adjusted LTL. Cocker spaniels, a medium sized breed, had a mean age adjusted LTL of more than 10kb less than the Labrador retrievers.

There was a significant difference in age adjusted RTL between breeds, although the above findings suggest that this was due to differences in factors such as cephalic index and inbreeding rate, rather than size or lifespan, as might have been originally anticipated.

It is unclear why RTL was so long in miniature schnauzers. The values for this breed were excluded from the analyses as the RTLs were greater than two standard deviations above the mean. Two miniature schnauzer samples were measured by TRF analysis and were within the range of the other samples. The HBD C_T s were not significantly different to the other breeds, but the C_T s for the telomere plate were lower. It may be possible that the telomere primers are annealing elsewhere in the genome as well as to the telomere in this breed. The efficiency of a standard curve generated using miniature schnauzer DNA could be checked using the same primer conditions as normal to see if the telomere reaction has very high efficiency.

Linear models showed that haematological and biochemical measures seemed to have little association with RTL. This may be in part due to acute changes of parameters while the dog is in hospital, such that the measures do not reflect long term organ function. One parameter which had highly significant relationship to RTL was neutrophil to lymphocyte ratio. This ratio has recently been reported as

being increased in dogs with soft tissue sarcoma compared to dogs with benign soft tissue tumours (L. Macfarlane *et al.*, 2016). The relationship between neutrophil to lymphocyte ratio and RTL may reflect the poor health of the dogs sampled; when higher ratios were considered, regression to RTL was significant. Previously, it has been demonstrated that different types of leukocyte have different telomere lengths (e.g. Robertson *et al.* 2000). The proportion of each present and therefore extracted from may have therefore had an impact upon the RTL. One option to overcome this is to separate out the different white cell types, using flow cytometry or a product such as Lymphoprep, and measure the telomere lengths separately. A problem would be potential poor recovery of granulocytes 24 hours after sample collection (Baerlocher *et al.*, 2006) and the blood samples obtained from the clinical pathology service were available 8 days after sample collection. However, neither neutrophil to lymphocyte ratio or lymphocyte to monocyte ratio were included in the final models. All four platelet variables were included (platelet count, mean platelet volume, plateletcrit and platelet distribution width) in model1, as well as calcium and monocyte count and PDW in model2. Only two studies could be found where platelet count was positively correlated with LTL (Hao *et al.*, 2005; Kozlitina and Garcia, 2012). No studies could be found regarding LTL and other platelet variables.

Several of the variables included in the final models for the qPCR optimisation samples are involved in inflammatory pathways, including cholesterol, calcium, monocytes and platelets. As discussed in section 1.7, telomere length is affected by oxidative stress. The pathways will not be discussed in detail as they are out of the remit for this thesis. Briefly, increased cholesterol concentrations lead to an inflammatory response through cholesterol accumulation in macrophages and other immune cells (Tall and Yvan-Charvet, 2015). Leukocyte-platelet interactions have been well-documented, and have shown to be relevant for inflammation and thrombosis. Activation of leukocytes through inflammation can lead to a procoagulant state (Afshar-Kharghan and Thiagarajan, 2006; Rumbaut and Thiagarajan, 2010). Moritz *et al* (2005) found that MPV and PDW, but not platelet count, were increased in dogs with both septic and non-septic inflammation

compared to healthy dogs. In addition, dogs with untreated multicentric lymphoma were found to have significantly greater platelet aggregation than control dogs (Thomas and Rogers, 1999). There is also platelet variability between breeds, which may explain why more platelet variables were included in model1, where breed was included. Greyhounds have been shown to have lower platelet count than non-greyhounds (Sullivan, Evans and McDonald, 1994) and Cavalier King Charles Spaniels had higher platelet aggregation response than Cairn Terriers, Boxers and Labrador retrievers (Nielsen *et al.*, 2007). Cavalier King Charles spaniels are prone to an inherited giant platelet disorder. In blood from 69 clinically normal Cavalier King Charles Spaniels, thrombocytopenia was found in 51.43% and macrothrombocytes were present in 33.33% (Cowan *et al.*, 2004). A reduction in albumin to globulin ratio is likely to represent a fall in albumin levels, which is often seen in chronic disease states (Moshage *et al.*, 1987) and serum albumin acts as a major antioxidant (Cha and Kim, 1996). The inclusion of sodium to potassium ratio in both models may represent a disease element due to the study population used. Reduced sodium to potassium ratio in dogs may be found in multiple disease states such as renal failure, pancreatitis, hypoadrenocorticism and heart failure (Roth and Tyler, 1999).

An issue of relating RTL to haematological and biochemical measures is the length of time between pathology testing and DNA extraction. Stability of complete blood count over time has been investigated. However, nearly all studies looked at stability of measures up to 72 hours, and the samples used here were stored for far longer. Hedberg and Lehto (2009) found that when samples were stored at 4°C, as they are in the diagnostics laboratory, all complete blood count parameters remained stable for 72 hours, except a slight increase in mean platelet volume. Interestingly, as previously mentioned, RTL has been demonstrated to be stable in EDTA-anticoagulated whole blood samples for up to 4 days (Zanet *et al.*, 2013). Whether this means the white cell differential becomes unstable at this point cannot be known without further study.

As the neutrophil to lymphocyte ratio to RTL regression was significant, numbers for the neutrophil and lymphocyte counts for the dogs used in the leukocyte TRF analysis were obtained. There was not a significant correlation between TRF and neutrophil to lymphocyte ratio ($R^2=0.09$, $p=0.33$). However, inclusion of neutrophil to lymphocyte ratio as an explanatory variable along with diagnosis of malignant disease and sex improved the linear model to TRF ($F=22.87_{(4,8)}$, $R^2=0.92$, $p<0.001$). Age was still not statistically significant as an explanatory variable for TRF.

There was no difference seen in LTL between entire and neutered dogs. It may be that neutering does not make a difference in LTL, but there was also no information on how long the dogs had been neutered for so it is not known if there was a time-dependent effect. There was also no relationship between LTL and index of deprivation. A reason that there was not an effect found may be that the sample was not representative of all backgrounds, given that the dogs from which the blood samples were taken were being treated at a referral centre.

4.4.3 Telomere length in oral samples

Another key aspect of this chapter was to consider whether a non-invasive sample could be used to measure representative telomere length in dogs. Buccal and saliva samples from Labrador retrievers were used. Buccal RTL regression to age was not initially significant, but the model was highly significant when neutering status were added to the model. This set of samples tested had the advantage of being from healthy dogs so the results were not affected by the presence of serious illness in the dogs. There was a significant regression between saliva RTL and age, which was also strengthened by the addition of sex into the model. Entire males had shorter saliva RTL, yet leukocyte RTL in these animals was not shorter. There was a significant regression between leukocyte and saliva RTL in matched samples, but only when sex was corrected for. The results show that saliva or buccal samples could be used in future dog studies, but there is a strong sex effect which needs to be corrected for. It would be helpful to perform

matched buccal and leukocyte RTL measurements to see if saliva or buccal telomere length correlated better with LTL.

It is not obvious why there was a sex effect on the oral telomere length measurements which was not present in the Edinburgh leukocyte samples. No sex difference was found in buccal telomere length in 109 children (Drury *et al.*, 2012) or in saliva telomere length in 89 adults aged 19-69 years (Chen *et al.*, 2015). One possibility for sex effecting oral telomere length is difference in dental disease prevalence between sexes. However, in beagles there was no sex difference in tooth clinical attachment due to dental disease (Kortegaard, Eriksen and Baelum, 2008). In addition, Gad (1968) found no sex difference in tooth cleanliness in 62 mongrels. No studies could be found regarding sex difference in dental disease in Labrador retrievers. Bender *et al* (2006) found oral neutrophil count was significantly higher in human patients with periodontal disease compared to controls. In the qPCR optimisation samples, neutrophil to lymphocyte ratio was seen to have a significant relationship with RTL; therefore, differences in white cell content could be present in each sex.

Limited information was available on the dogs from whom the saliva and buccal samples were taken, so it is not known if factors such as weight affected oral telomere length. In addition, only one breed was used so it is not known if the factors that affected leukocyte RTL, such as cephalic index and inbreeding, affect oral telomere length.

4.4.4 Chapter summary

The main focus of this chapter was to optimise the qPCR methodology for telomere length measurement in dog samples. The method was used to demonstrate age related decline in LTL successfully. However, factors such as presence of malignancy in the individual, breed, cephalic index and inbreeding affected the regression, so the picture was not straight forward. In addition, DNA sample

quality and extraction method were shown to have an impact upon LTL and these factors need to be considered in future experiments.

There was a significant association observed between samples measured by qPCR and the “gold standard” telomere measurement technique, TRF analysis. However, the relationship was not strong and this may have been due to the TRF technique not being fully optimised for dog sample measurement. An absolute telomere length qPCR technique was attempted, but decided against after highly variable results were generated. Thus, subsequent telomere length measurements were made using the relative qPCR method. The relative qPCR method was optimised and was used to demonstrate age related changes in groups of samples, and thus was deemed suitable to measure telomere length in Chapter 5.

Buccal and saliva samples were tested for their possibility of use for telomere length measurement and showed promise in this area, with the caveat that sex, including neutering status, had a large association with results. Further investigation is warranted into this area.

Chapter 5: Evaluating the relationship between environmental tobacco smoke and telomere length

5.1 Introduction

5.1.1 Smoking and telomere length

No documented evidence of the effect of ETS on canine telomere length could be found in the literature. Therefore, evidence from human studies, and murine studies, will be presented here. Studies on the relationship between smoking and telomere length in humans were found via PubMed and Ovid Medline using the search terms “cigarette”, “smok*” or “tobacco” and “telomere”. A total of 216 studies were found. However, in only a small number of these is smoking a main focus of the study. Examples of studies where investigating the association between smoking and telomere length is one of the key aims of the study are given below.

5.1.2 Smoking and cross-sectional leukocyte telomere length

Valdes et al. (2005) examined leukocyte TRF length in 1122 white females aged 18 to 76. They found current smokers had the shortest TRF lengths and a dose-dependent relationship existed between smoking and leukocyte TRF length, with an additional 18% of LTL lost over and above the average attrition rate per pack-year smoked. Latifovic et al. (2016) measured LTL using qPCR in 477 healthy volunteers aged 20 to 50. Again, current daily smoking was associated with significantly shorter LTL than never smokers. However, while those with >0 to 6.25 and >6.35 to 16 tertiles of pack-years smoking had shorter LTL than never smokers, the group with greater than 16 pack-year smoking history did not have significantly different LTL to never smokers. In a third study, leukocyte TRF length was measured in 305 adults, with mean age 42.5, randomly recruited from a Flemish community. After adjustment for age, TRF length was significantly shorter in smokers than non-smokers in this study too, and TRF decreased with increasing number of pack years (Nawrot *et al.*, 2010). Strandberg et al. (2011) measured leukocyte TRF length in 622 men, of mean age 75.7, partaking in a follow-up of the Helsinki Businessmen Study. They also calculated the proportion of telomeres less than 5kb in length in each sample. Age-adjusted TRF was again significantly longer

in never smokers than past or current smokers. There also tended to be a higher proportion of short telomeres in former or current smokers than non-smokers. Morlá *et al.* (2006) measured lymphocyte telomere length in 26 never-smokers of mean age 57, 24 smokers with normal lung function with mean age 58 and 26 smokers with COPD of mean age 61 using FISH. There was a negative association between pack year and telomere length, which was not affected by the degree of airflow obstruction present. Verde *et al.* (2015) measured LTL in 147 healthy smokers with mean age 43.2. Pack years of smoking were associated with an increased odds ratio of having short LTL. However, no significant correlation was observed between LTL and urinary nicotine concentration or between LTL and urinary cotinine concentration. They also calculated the nicotine metabolism ratio (the ratio of trans-3'-hydroxycotinine to cotinine), which reflects P450 2A6 activity. The nicotine metabolism ratio was also unrelated to LTL.

Two of the studies examining smoking and telomere length were case-control studies. Mirabello *et al.* (2009) examined risks for prostate cancer in men aged 55 to 74. The study included 612 cases and 1049 matched controls. LTL was measured by qPCR. They examined the association between smoking and LTL in the cases and controls combined, and found a significant inverse correlation between LTL and pack-years smoked. However, when they separated the cases and controls, there was only a significant relationship between pack-years and LTL in controls, not cases. McGrath *et al.* (2007) examined LTL, cigarette smoking and bladder cancer risk, using 184 cases and 192 controls from the Nurses' Health Study and Health Professional Follow-up Study (HPFS). They observed a significant difference in LTL across pack-years of smoking, adjusted for age and gender, but only an association between LTL and pack-years of smoking in HPFS control subjects when the subjects were divided into studies and cases and controls.

5.1.3 Smoking and longitudinal leukocyte telomere length

Huzen *et al.* (2014) measured LTL using qPCR in 8074 participants aged 28 to 75 in an ongoing community based prospective cohort study. LTL was measured 1 to 3

times over an average period of 6.6 years. Active smoking tripled the average LTL attrition rate per year. In another study, 4576 individuals from the Copenhagen City Heart Study, aged 40 to 47 at baseline, gave blood samples at two time points 10 years apart. LTL was measured by qPCR. Current smoking was associated with shorter LTL at time point 1 only, and daily tobacco consumption was not associated with LTL at either time point. Increased baseline tobacco consumption was actually associated with an increase in LTL base pairs between the two time points in a univariate analysis, but there was no significant association between tobacco consumption and LTL change in multivariable analyses when variables such as age were included (Weischer, Bojesen and Nordestgaard, 2014). Müezziner *et al.* (2015) measured LTL in a subset of an ongoing cohort study using qPCR. LTL was measured at baseline in 3600 participants aged 50-75 and repeated in 1000 participants after 8 years. At baseline, current smokers statistically had the shortest LTL, and a trend was seen in LTL shortening with increasing cigarettes per day. Conversely, there was a non-significant trend for never smokers to experience greater attrition in LTL per year compared to smokers between the two time points, except in those who smoked 20 or more cigarettes per day.

5.1.4 Smoking and telomere length in other tissues

The majority of telomere length studies published measure telomere length in blood, but in this thesis other tissues were examined as well as blood. Chen *et al.* (2015) measured telomere length in saliva using qPCR in a group of 89 caregivers aged 19 to 69. They found that current smokers had significantly shorter saliva telomere length compared to former or never smokers. Broberg *et al.* (2005) studied the risk of bladder cancer based on buccal cell telomere length, and investigated the impact of smoking on this risk in 63 bladder cancer cases aged 35 to 85 and 93 controls aged 21 to 90. No significant difference in buccal cell telomere length was found between current and non-smokers (including former smokers). However, the people at the greatest risk of bladder cancer were current smokers with short buccal cell telomere length. In another study, small airway epithelium was collected in 29 healthy lifelong non-smokers (mean age 38) and 29 healthy smokers (mean age 38.8). Smoking status was verified by urinary

nicotine and cotinine concentrations. Small airway epithelium telomere length was measured by TRF analysis. Initially, no association between TRF length and pack-year history was found, but when the data was subsetted by race, TRF shortening with increasing pack-years was observed, with the relationship being statistically significant in the white population. Leukocyte TRF length was also measured in a subset of the study population. No difference was observed in leukocyte TRF length between smokers and non-smokers, yet in this subpopulation there was a significant difference in small airway epithelium TRF length between smokers and non-smokers (Walters *et al.*, 2014).

A recent study examined the impact of polycyclic aromatic hydrocarbons (PAHs), a component of cigarette smoke, on sperm telomere length. 666 human male volunteers provided semen for telomere length analysis and urine for measurement of eight PAH metabolites. Two of the metabolites were significantly related to a decrease in sperm telomere length, but the other six metabolites were not associated with telomere length (Ling *et al.*, 2016).

5.1.5 Study related factors and relationship between smoking and telomere length

The 216 studies found where smoking was investigated as a potential explanatory variable for telomere length are listed in a table in Appendix 9. It was noted if telomere length measurements in each study were cross sectional or longitudinal, the method of telomere length measurement and coefficient of variation for the method, the tissue used for telomere measurement and DNA extraction method. In terms of study participants, the number of individuals with both telomere length measurement and smoking history, percentage of participants who were current smokers, average participant age, sex, notable chronic health problems and if the smoking status was recorded in categories or numerically were tabulated. It was also recorded if there was a significant relationship between telomere length and age. Finally, it was recorded if smoking was found to have a significant relationship with telomere length, taking significance as $p < 0.05$. Where

components were not explicitly stated, if possible an estimate was made using the information in the publication, if not, “n/a” was entered in the cell. Some studies involved a subset of a larger cohort study, which was used in several publications. Where studies used the same cohort, these were grouped in the table.

99 studies found a significant relationship between smoking and telomere length. Six of these studies found a positive association between pack year history and LTL or reduced loss over time in smokers; all others demonstrated a negative relationship between pack year history and telomere length and/or a reduction in telomere length based on smoking status.

5.1.6 Environmental tobacco smoke exposure and telomere length

The majority of studies found on the association of ETS exposure with telomere length focussed on prenatal exposure. For example, Salihu *et al.* (2015) assessed foetal telomere length by qPCR, using cord blood leukocytes from 86 individuals. Statistically significant differences were found between smoke exposure groups. Telomere length was shortest when the mother actively smoked during pregnancy, followed by the group of non-smoking mothers exposed to ETS, and was longest in non-smoking mothers not exposed to ETS. Imam *et al.* (2012) collected various perinatal tissues from 103 mothers with infants including: maternal venous blood, maternal blood spot, infant heel prick blood, placental tissue from both maternal and foetal sides, and cord blood. Telomere length was measured in all tissues by qPCR. Telomere lengths were then compared based on maternal smoking status. Cord blood telomere length was significantly shorter in infants exposed to maternal smoking. Maternal side placenta telomere length was also significantly shorter in smoking mothers, but only when other factors were adjusted for. No significant differences were found in the other tissues based on maternal smoking behaviours. In a third study, 169 cord blood samples were collected and T cell telomere length was measured using qPCR. Smoking status of the mother was obtained by questionnaire and confirmed by maternal venous and cord blood cotinine concentrations. In contrast to the findings by Imam *et al.* 2012 and Salihu

et al. 2015, cord blood T lymphocyte telomere length was significantly longer in new-borns of smoking mothers.

Further studies investigated the impact of retrospective prenatal ETS exposure on telomere length in children. For example, Pawlas *et al.* (2015) examined LTL in 99 Polish 8-year-old children, using qPCR. They found that LTL was significantly shorter in children whose mothers smoked during pregnancy. Furthermore, when saliva telomere length was measured in 104 children aged 4 to 14 years, saliva telomere length was significantly shorter in children with a prenatal tobacco smoke exposure history (Theall *et al.*, 2013). In contrast, when LTL was measured in 331 Australian children (with mean age 3.6 years) by qPCR, there was no significant difference in telomere length based on maternal smoking during pregnancy (Skilton *et al.*, 2016). Ip *et al.* (2016) measured buccal cell telomere length using qPCR in 196 children of mean age 6.33. Like Pawlas *et al.* 2015 and Theall *et al.* 2013, telomere length was shorter in children whose mothers smoked throughout pregnancy. However, they found no association between maternal ETS exposure during pregnancy and child telomere length, or between post-natal ETS exposure and telomere length.

5.1.7 Murine telomere length and tobacco smoke

Murine experiments regarding ETS exposure and telomere length are limited, perhaps due to the difference in human and mouse telomeres, or the availability of clinically obtained human samples demonstrating the association between ETS and telomeres. It should be noted that all the following studies were written by the same author. In experiments investigating the effect of cigarette smoke on embryo development *in vivo*, female CD1 mice were exposed to cigarette smoke, given doses of cigarette smoke condensate or untreated. They were mated and their embryos harvested after 4 days. While telomere length was significantly shortened in the embryos of mice exposed to cigarette smoke or cigarette smoke concentrate compared to no exposure, as measured by Q-FISH, there was no change in the rate of chromosomal abnormalities or fusions (Huang *et al.*, 2009).

In further experiments, CD1, B6C3F1 or telomerase RNA component (*Terc*^{-/-}) knockout C57B1/6 female mice were superovulated using PMSG (pregnant mare serum gonadotrophin), followed by hCG (human chorionic gonadotrophin) and then mated. Zygotes were collected, cumulus cells removed and embryos cultured. The embryos were exposed to cigarette smoke condensate or cadmium (an abundant compound in tobacco). Both exposures resulted in an increase in oxidative stress and shorter telomere length, measured by Q-FISH. Significantly more telomere loss occurred in the *Terc* knockout mice than in the wild-type mice. In addition, cigarette smoke condensate resulted in loss of telomere free ends and chromosome fusion (Huang *et al.*, 2010). Similar experiments were conducted on mouse embryonic stem cells. Mouse embryonic stem cells were isolated from C57 mice and cultured. The cells were treated with cigarette smoke condensate or cadmium, or were controls. Telomere lengths of cells treated with cigarette smoke concentrate were consistently shorter than those of control cells. Cells treated with cadmium had the shortest telomere lengths. Cells treated with low dose cadmium or cigarette smoke concentrate demonstrated the loss of telomere free ends and chromosome fusion (Huang *et al.*, 2013).

5.1.8 Chapter aims and hypotheses

The key aim of this chapter was to explore whether there was an association between ETS exposure and telomere length. However, to achieve this, the correlation between telomere lengths in individuals was tested, to establish if one measure of telomere length would reflect telomere lengths elsewhere in the body. In addition, as telomere length was being viewed as a biomarker of ageing, tissues and the differences between pairs of tissues were tested to see which would be the best representation of dog age. Using hair nicotine and cotinine as objective measures of ETS, it was then aimed to explore whether either of these markers were associated with telomere lengths both cross-sectionally at both time points, and longitudinally between the two time points. In addition, further analyses were undertaken to explore whether other variables impacted upon telomere length.

It was hypothesised that tissue telomere lengths would be highly correlated within individuals. As cremaster muscle is generally considered to be a post-mitotic tissue, cremaster muscle telomere length was expected to be the longest telomere length measure and to not be associated with age. In addition, vas deferens telomere lengths were not expected to significantly change with age, unless a trauma was reported to the area, as smooth muscle cells are capable of repair. Epididymis telomere length was hypothesised to increase with age, as a proxy for sperm telomere length. Leukocyte and buccal telomere lengths were expected to shorten between the time points, and to be negatively associated with age. In addition, those with longer leukocyte and buccal telomere lengths at time point 1 were expected to have larger decreases in telomere length over the study period.

It was also expected that dogs exposed to ETS would have shorter leukocyte and buccal telomere lengths cross-sectionally, and that these dogs would experience greater rates of telomere attrition. The difference between leukocyte and cremaster muscle telomere lengths was expected to increase with age, and to increase with exposure to ETS, and the rate of change between these tissues was expected to decrease with age but increase with ETS. These age-related changes were also expected for the difference between leukocyte and vas deferens telomere lengths. However, as it is not a post mitotic tissue, damage from ETS may be evident in vas deferens telomere shortening. It was predicted that the difference between LTL and epididymis telomere length would increase with age at a faster rate than the differences between the other tissue pairs, with epididymis telomere length increasing with age and LTL decreasing with age. However, it was also expected that ETS would cause damage to epididymis telomeres and these would shorten with increasing exposure to ETS.

In the additional analyses, it was hypothesised that weight and height would be negatively associated with telomere length in all tissues. Males were expected to have shorter leukocyte and buccal telomere lengths than females. Breed life expectancy was expected to be positively associated with telomere lengths, and

those with a shorter life expectancy to show an increased rate of loss in telomere length between the two time points.

5.2 Methods

5.2.1 Study participants

To decide upon the number of dogs required to investigate the association between ETS and telomere length, a power analysis was conducted using the limited published data. Calculations were based on the mean leukocyte - muscle telomere length in dogs being -1.25 ± 0.65 kb (using data from Benetos *et al.* 2011) and exposure to environmental tobacco smoke (ETS) causing the same level of damage to dog telomeres as smoking in humans (using data from Valdes *et al.* 2005). An age matched design was assumed and it was calculated that a study with 16 dogs in both ETS exposed and non-ETS exposed groups would have 80% power to detect a statistically significant difference (at $p < 0.05$) in leukocyte - muscle telomere length. Given the uncertainties in the calculations, 20 per group seemed prudent. As described in section 2.2.9.2, 2 further dogs were added after discovering that 2 dogs had been rehomed soon after the first study time point, resulting in the 42 dogs described in section 2.2.9.5.

5.2.2 Samples

Samples were collected as described in section 2.2.9.1. Blood haematology and biochemistry results were collated for both time points. One of the cavalier King Charles spaniels was found to have giant platelet disorder, so the platelet data for this dog were excluded from analyses. 4 samples had biochemical results indicating EDTA contamination, and therefore the potassium and calcium concentrations for these were excluded as well.

Dog weight was recorded at both time points and the percentage weight change calculated. To account for expected weight gain due to growth, growth curves were used to calculate expected final adult weight in the dogs which appeared to have not finished growing. The percentage difference between the expected adult weight and weight at time point 2 was also then calculated. Breed height and

cephalic index were as in Chapter 3. The estimated effective population size for breeds were obtained from the Kennel Club UK website (The Kennel Club Limited, 2016). Due to the nature of this measure, there were no values for cross breeds. In addition, no values for Patterdale terrier or Shar-pei were present on the website. The value for Parson Russell terrier was assigned to the Jack Russell terrier. Breed life expectancy was obtained using figures from Adams et al. 2010, Michell 1999 and O'Neill et al. 2013. Life expectancy figures were not available for specific cross breeds, or for the Patterdale terrier.

Questionnaire and hair analysis data were as in Chapter 3. Using their postcodes, owner indices of deprivation were obtained from the Scottish Neighbourhood Statistics website (The Scottish Government, 2015b).

5.2.3 Telomere length measurement

DNA was extracted from blood and buccal swabs using a column extraction method as detailed in section 2.2.2. DNA was extracted from cremaster muscle, epididymis, vas deferens and uterus samples using a phenol-chloroform method as described in section 2.2.1. DNA quantity and quality were measured using spectrophotometry as per section 2.2.5, ensuring that the minimum quality control standards were reached for each DNA sample. If the quality was deemed unacceptable, either the extraction process was repeated or the DNA sample cleaned until an acceptable standard was reached. Relative telomere length was measured by qPCR, using the optimised protocol from chapter 4. DNA samples from dogs from both smoking and non-smoking homes were analysed on each plate to reduce the chance of finding a difference in RTL between the exposure groups because of inter-assay variation.

5.2.4 Data analysis

5.2.4.1 Smoking and telomere length studies

Binomial generalised linear regression was used to compare the outcomes of existing telomere studies assessing the impact of smoking based on study design features. If a significant association was found, the study was assigned a “1”. If no significant association between smoking and telomere length, the study was assigned a “0”. Different elements of study design such as method used to measure telomere length were then input into the model as explanatory variables.

5.2.4.2 Longitudinal RTL measurements

The differences between RTLs at time points 1 and 2 were taken for each dog. As some participants did not attend the clinic at appointments exactly a year apart, the change in RTL was adjusted by dividing the number of days between appointments by 365.25 and multiplying this number by the change in RTL. This calculation assumed a constant daily rate of change. When comparing the change in leukocyte and buccal RTLs to the time point 1 values, the change was corrected for regression to the mean using the formula given in Verhulst *et al.* (2013):

$$D = p(X_1 - \bar{X}_1) - (X_2 - \bar{X}_2)$$

where

$$p = \frac{2rS_1S_2}{S_1^2 + S_2^2}$$

$$S^2 = \text{variance}$$

5.2.4.3 Comparison between postmitotic and proliferative tissue RTL

Comparisons between tissue telomere lengths were as per the model proposed by Benetos *et al.* (2011) (see Chapter 1). The basis of using a comparative tissue is that a post-mitotic tissue would reflect telomere length at birth, so by subtracting leukocyte or buccal telomere length the loss since birth would be reflected. By

then dividing by age in months, this would give the rate of loss per month since birth (based on a constant rate of loss). For the tissue telomere length comparison analyses, the difference was calculated simply by subtracting either cremaster muscle RTL, epididymis RTL or vas deferens RTL from leukocyte RTL or buccal RTL. To calculate the rate of change between the tissues, leukocyte or buccal RTL was subtracted from cremaster muscle, vas deferens or epididymis RTL and then divided by the dog age in months. As there is only one point at which the dogs were castrated, for obvious reasons, time point 2 leukocyte and buccal telomere lengths were compared to the telomere lengths of tissues obtained at time point 1.

5.2.4.4 Statistical testing

Linear modelling analysis was as described in chapter 4 methods. Paired Wilcoxon rank sum tests were used to compare RTL measurements between tissues. Pearson product moment correlation was used to test the relationship in RTL in different tissues.

As not all breeds had the same life expectancy, life expectancy (LE) adjusted age was calculated by dividing age by life expectancy.

5.3 Results

5.3.1 Study design in publications

Binomial generalized linear regression was employed to see if there were any factors in study design which impacted upon finding a significant relationship between telomere length and smoking, in the 216 studies in Appendix 9.

As may be expected, having an increased number of individuals in the study resulted in an increased likelihood of finding a relationship between telomere length and smoking. In addition, having a higher proportion of smokers had a similar effect. If fewer than 25% of participants were current smokers, an effect was less likely to be found and if more than 50% of the group were current smokers, a relationship was more likely to be found. There was a tendency for a relationship being less likely when the average age of the participants increased, perhaps due to other factors such as ill health confounding. This was compounded by the increased tendency to find an effect in studies where the participants were “healthy”. No sex effect was found on the likelihood of finding an association. Aside from the salting-out DNA extraction method tending to have a negative impact on the likelihood of finding an association between smoking and telomere length, there was no effect of DNA extraction and measurement method or coefficient of variation seen. However, quite a few studies did not state DNA extraction method or coefficient of variation. If a significant relationship was seen between age and telomere length in a study, a relationship between smoking and telomere length was significantly more likely to be found. Longitudinal telomere measurements did not make finding an association more likely. Finally, an effect was more likely to be found if a numerical measure of smoking was included, rather than just smoking status.

An interesting observation on these publications is that some use subsets of participants from the same larger studies, but the result can be different. For example, 4 studies used participants from the Prevention of Renal and Vascular End-Stage Disease Study. 2 found a significant relationship between smoking and telomere length, 2 did not (Kingma *et al.*, 2012; Huzen *et al.*, 2014; van

Ockenburg *et al.*, 2014, 2015). The sub-cohorts were similar in age and smoking rate and telomere lengths were measured by qPCR in all, yet the findings were not the same. Studies using the same larger study are grouped together in Appendix 7 for comparison.

5.3.2 Study dogs

Table 5-1 gives the ages, weights and smoking variables associated with each breed at time point 1, and Table 5-2 provides this information for time point 2. Given the wide range of breeds in the study, breeds were sorted into 4 groups to ease analysis: terrier/terrier cross, collie/collie cross, spaniel/spaniel cross and other (Table 5-3 and Table 5-4). As can be seen in Table 5-1, there were no dogs which could fall into two groups. The Staffordshire bull terrier breed was the only breed with any females.

Table 5-1 Dog breeds in the study at time point 1 with associated variables with age, weight, HNC in ng/mg and HCC in ng/mg. Where more than two dogs of a breed are present, the range of values and median are given.

Breed	Number	Age	Weight	Nicotine	Cotinine
Basset hound	1	0.76	16.6	2.44	0.14
Beagle	1	0.74	14.5	0.03	0.05
Bearded collie	1	2.33	18.4	0.27	0.05
Border collie	4	0.65 (0.61-1.1)	16.7 (13.1-19.5)	0.08 (0.01-1.08)	0.01 (0-0.01)
Border terrier X Pug	1	0.62	9.3	0.08	0.06
Boxer	1	2.1	33.1	0.20	0.08
Cavalier King Charles spaniel	2	0.62, 0.70	6.3, 9.2	4.05, 5.93	0.22, 0.62
Cocker spaniel	1	0.53	10.5	0.03	0.02
Collie X Labrador retriever	1	0.47	13.6	1.79	0.24
German shepherd dog X collie	1	1.42	24	0.01	0
German shorthaired pointer	1	6.58	31.2	0.09	0
Jack Russell terrier	1	0.72	5.9	0.02	0
Miniature schnauzer	1	6.16	9.1	0.01	0
Patterdale terrier	1	5.33	12.8	0.01	0
Patterdale terrier X Lakeland terrier	1	0.51	12.3	0.44	0.04
Pomeranian X Border terrier	1	0.51	5.1	0.02	0
Retriever X Rottweiler/Bull mastiff	1	0.46	18.8	0.08	0

Rottweiler	1	1.36	44.2	0.16	0.05
Shar-pei	1	2.83	11	0.06	0
Springer spaniel	2	0.53, 2.19	17.2, 19.8	0.02, 0.36	0, 0.05
Springer spaniel X German shepherd dog	1	0.51	20.4	0.06	0
Staffordshire bull terrier	12 (5F, 7M)	2.33 (0.57- 5.09)	18 (13.5- 20.8)	0.14 (0.01-1.73)	0.08 (0-0.678)
Staffordshire bull terrier X Lurcher	2	2.84, 2.84	19.9, 27.5	0.45, 1.73	0.13, 0.15
Yorkshire terrier	2	4.40, 7.04	6.6, 7.3	0.81, 13.80	0.19, 0.87

Table 5-2 Dog breeds in the study at time point 2 with associated variables**Where more than two dogs of a breed are present, the range of values and median are given.**

Breed	Number	Age	Weight	Nicotine	Cotinine
Basset hound	1	1.68	21.7	1.94	0.28
Bearded collie	1	3.42	25.7	0.10	0
Border collie	4	1.72 (1.67-2.10)	18.4 (15.6-22.9)	0.13 (0.01-0.36)	0.10 (0-0.42)
Border terrier X Pug	1	1.66	12.2	0.15	0.05
Cavalier King Charles spaniel	2	1.73, 1.86	9.5, 11.6	9.62, 12.59	0.35, 0.48
Cocker spaniel	1	1.59	12.3	0.02	0
Collie X Labrador retriever	1	1.56	21	0.97	0.20
German shepherd dog X collie	1	2.46	24.9	0.01	0
German shorthaired pointer	1	7.59	31.8	0.02	0
Jack Russell terrier	1	1.71		0.03	0
Miniature schnauzer	1	7.25	11	0.01	0
Patterdale terrier	1	6.24	15.4	0.01	0
Patterdale terrier X Lakeland terrier	1	1.52	15.5	0.33	0
Pomeranian X Border terrier	1	1.64	6.5	0.04	0
Retriever X Rottweiler/Bull mastiff	1	1.54	32.3	0.06	0

Springer spaniel	2	1.40, 3.24	23.3, 26.9	0.01, 0.20	0, 0.21
Staffordshire bull terrier	2 (1M, 1F)	1.40, 5.73	16.5, 17.8	0.02, 0.04	0, 0
Staffordshire bull terrier X Lurcher	1	3.83	36.2	1.31	0.71
Yorkshire terrier	1	5.27	7.2	0.09	0.08

**Table 5-3 Dogs in the study at time point 1 arranged into four breed groups
With median and ranges of variables**

Breed group	Number	Age	Weight	Nicotine	Cotinine
Collie/CollieX	7	0.65 (0.47-2.33)	18.4 (13.1-24)	0.14 (0.01-1.79)	0.01 (0-0.24)
Spaniel/SpanielX	6	0.58 (0.51-2.19)	13.9 (6.3-20.4)	0.21 (0.02-5.93)	0.04 (0-0.62)
Terrier/TerrierX	21	2.57 (0.51-7.04)	15 (5.1-27.5)	0.29 (0.01-13.79)	0.07 (0-0.87)
Other	8	1.73 (0.46-6.58)	17.7 (9.1-44.2)	0.09 (0.01-2.44)	0.02 (0-0.14)

**Table 5-4 Dogs in the study at time point 2 arranged into four breed groups
with median and ranges of variables**

Breed group	Number	Age	Weight	Nicotine	Cotinine
Collie/CollieX	7	1.72 (1.56-3.42)	21.0 (15.6-25.7)	0.01 (0.01-0.97)	0 (0-0.20)
Spaniel/SpanielX	5	1.73 (1.40-3.24)	12.3 (9.5-26.9)	0.20 (0.01-12.59)	0.21 (0-0.48)
Terrier/TerrierX	9	1.71 (1.40-6.24)	15.4 (6.4-36.2)	0.04 (0.01-1.31)	0 (0-0.71)
Other	4	4.46 (1.54-7.59)	26.8 (11-32.3)	0.04 (0.01-1.94)	0 (0-0.28)

5.3.3 Tissue synchrony

Relative telomere length (RTL) was measured using qPCR in leukocytes and buccal samples from time points 1 and 2, as well as cremaster muscle, epididymis, vas deferens and uterus samples. As there were only five females, uterus RTLs were not used in further analyses.

RTL measurements greater or less than two standard deviations away from the mean for each tissue across the population were deemed outliers and were excluded. Of note of these exclusions were all measurements for the miniature schnauzer and a Border collie, aged 7 months at time point 1. The C_T values for the HBD PCRs for the Border collie were approximately four cycles higher than the average value for all dogs, resulting in very high RTL values for this dog. In addition, DNA yielded from 5 of the buccal swabs was of insufficient quantity and quality to use for qPCR analysis. After applying the exclusions, 40 leukocyte RTLs from time point 1 (LRTL1), ranging in value from 0.40 to 2.72, 23 leukocyte RTLs from time point 2 (LRTL2), ranging from 0.35 to 3.62, 39 buccal RTLs at time point 1 (BRTL1), ranging from 0.23 to 4.89, and 19 from time point 2 (BRTL2), with values from 0.48 to 6.10, remained for analysis. Due to the samples with insufficient DNA and RTL values outside of the accepted range, there were only 18 individuals with longitudinal buccal RTL measurements (measurements at both time points). 35 cremaster muscle RTLs (CMRTL) (ranging 0.38 to 4.75), 33 epididymis RTLs (ERTL) (0.47 to 5.24) and 34 vas deferens RTLs (VDRTL) (0.31 to 4.29) were available for analysis. 7 dogs had LRTL1 values which were longer than their CMRTL. In addition, 6 dogs had a longer LRTL2 than CMRTL, with 4 of these dogs also having longer LRTL1 than CMRTL too. 8 dogs had longer LRTL2 than LRTL1. One dog had a smaller percentage change than the inter-assay variation (i.e. less than 3.64%), and thus was labelled as a “maintainer”. BRTL2 was longer than BRTL1 in 11 out of the 18 dogs who had valid measures at both time points.

Figures 5.1 and 5.2 show the RTL measurements in tissues used for analyses.

Wilcoxon rank sum tests were performed to look for pairwise differences between

RTLs in tissues. CMRTLs were significantly longer than LRTL1s ($p<0.001$), LRTL2s ($p<0.001$) and BRTL1s ($p=0.03$), but not BRTL2s ($p=0.23$). Likewise, ERTLs were significantly longer than LRTL1s ($p=0.006$) and LRTL2s ($p=0.003$) but not BRTL1s ($p=0.17$) or BRTL2s ($p=0.87$). VDRTLs were not significantly different from LRTL1s ($p=0.20$), LRTL2s ($p=0.11$), BRTL1s ($p=0.66$) or BRTL2s ($p=0.68$). There were no significant differences between LRTL1s and LRTL2s ($p=0.32$), BRTL1s and BRTL2s ($p=0.35$) or LRTL1s and BRTL1s ($p=0.49$), but BRTL2s were significantly longer than LRTL2s ($p=0.02$). There were no significant differences between VDRTLs, CMRTLs and ERTLs ($p>0.05$ for all pairwise tests).

Following Shapiro-Wilk tests, LRTL1, LRTL2, BRTL1, BRTL2, ERTL and VDRTL values were log transformed to normalise their distributions. However, this resulted in negative values and so non-transformed values were used when examining the difference in RTL between tissues.

To investigate if telomere lengths were well synchronised within individuals, pairwise correlation tests using Pearson's product moment correlation coefficient were performed between tissues (Table 5-5). Significant positive correlations only existed between $\ln(\text{LRTL1})$ and $\ln(\text{BRTL1})$ ($p<0.001$), and $\ln(\text{VDRTL})$ and $\ln(\text{ERTL})$ ($p=0.047$). The correlations between LRTL1 and LRTL2 ($p=0.36$) and BRTL1 and BRTL2 ($p=0.67$) were not statistically significant (Figure 5.3 and Figure 5.4).

Table 5-5 Correlation coefficients between different tissue types* indicates $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

ln(LRTL2)	0.20					
ln(BRTL1)	0.53***	0.32				
ln(BRTL2)	0.43	0.04	0.11			
CMRTL	0.14	0.09	0.19	0.30		
ln(VDRTL)	0.04	0.14	0.16	-0.21	0.19	
ln(ERTL)	0.08	0.04	0.30	0.15	0.23	0.35*
	ln(LRTL1)	ln(LRTL2)	ln(BRTL1)	ln (BRTL2)	CMRTL	ln (VDRTL)

Figure 5.5 shows ladder plots of paired measures in individuals of CMRTL and LRTL1 (A), LRTL1 and LRTL2 (B) and BRTL1 and BRTL2 (C). The plots demonstrate that individuals with the longest RTLs at time point 1 or longest CMRTLs do not necessarily maintain their rank at time point 2 or between tissues.

It was next considered whether the difference in RTL measurements at time points 1 and 2 were dependent on the baseline measure at time point 1. The linear model of the difference between LRTL1 and LRTL2 to LRTL1 was statistically significant ($F=9.20$, $R^2=0.29$, $p=0.006$), indicating that the individuals with longer LRTL1 had increased attrition between the two time points. However, it has been hypothesised that this observation may be due to regression to the mean.

Therefore, the difference in RTL between the time points was corrected for this effect. The linear model was still statistically significant, although the relationship was weaker (Figure 5.6A, $F=7.28$, $R^2=0.25$, $p=0.01$). The process was repeated with the BRTL measurements from the two time points. Without correction for regression to the mean, the linear model of BRTL1-BRTL2 to BRTL1 was statistically significant ($F=9.32$, $R^2=0.37$, $p=0.008$), with again those with longer BRTL1 having a greater degree of telomere length loss by time point 2. After incorporating the regression to the mean correction, the linear model was

still statistically significant, although again the relationship was weaker ($F=5.38$, $R^2=0.24$, $p=0.03$, Figure 5.6B).

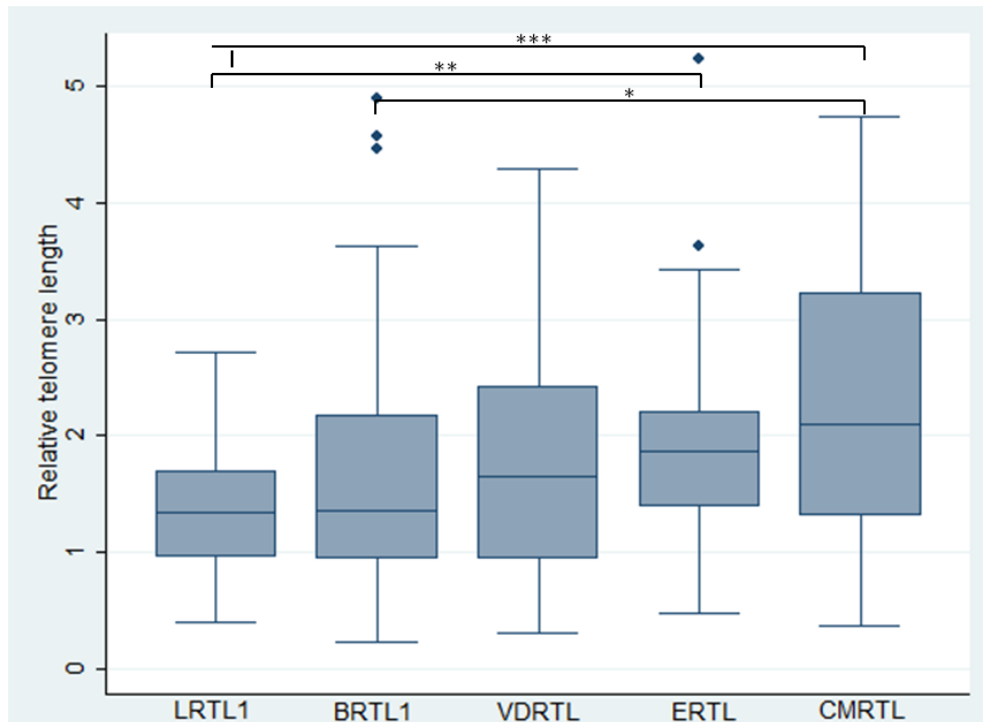


Figure 5.1 Relative telomere length in tissues at time point 1

Relative telomere lengths in leukocytes at baseline (LRTL1), buccal cells at baseline (BRTL1), vas deferens (VDRTL), epididymis (ERTL) and cremaster muscle (CMRTL). Significant pairwise differences identified using Wilcoxon rank sum tests are indicated by $*=p<0.05$, $**=p<0.01$ or $***=p<0.001$

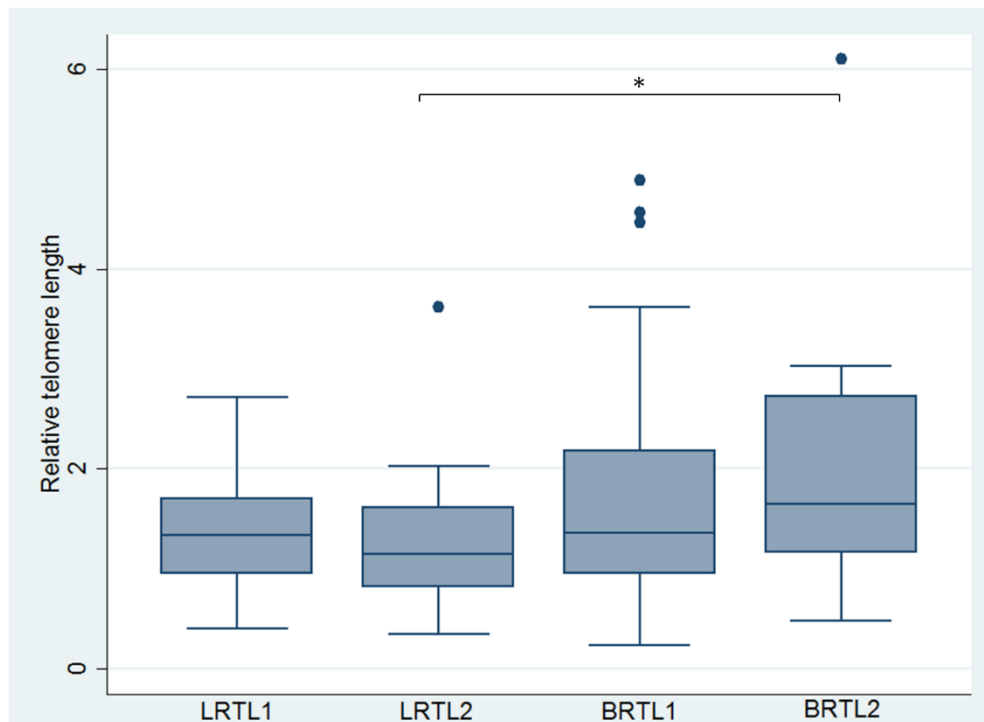


Figure 5.2 Relative telomere lengths in leukocyte and buccal samples

Relative telomere lengths in leukocytes at time point 1 (LRTL1) and 2 (LRTL2), and in buccal cells at time point 1 (BRTL1) and 2 (BRTL2). Significant pairwise differences identified using Wilcoxon rank sum tests are indicated by $*=p<0.05$, $**=p<0.01$ or $***=p<0.001$

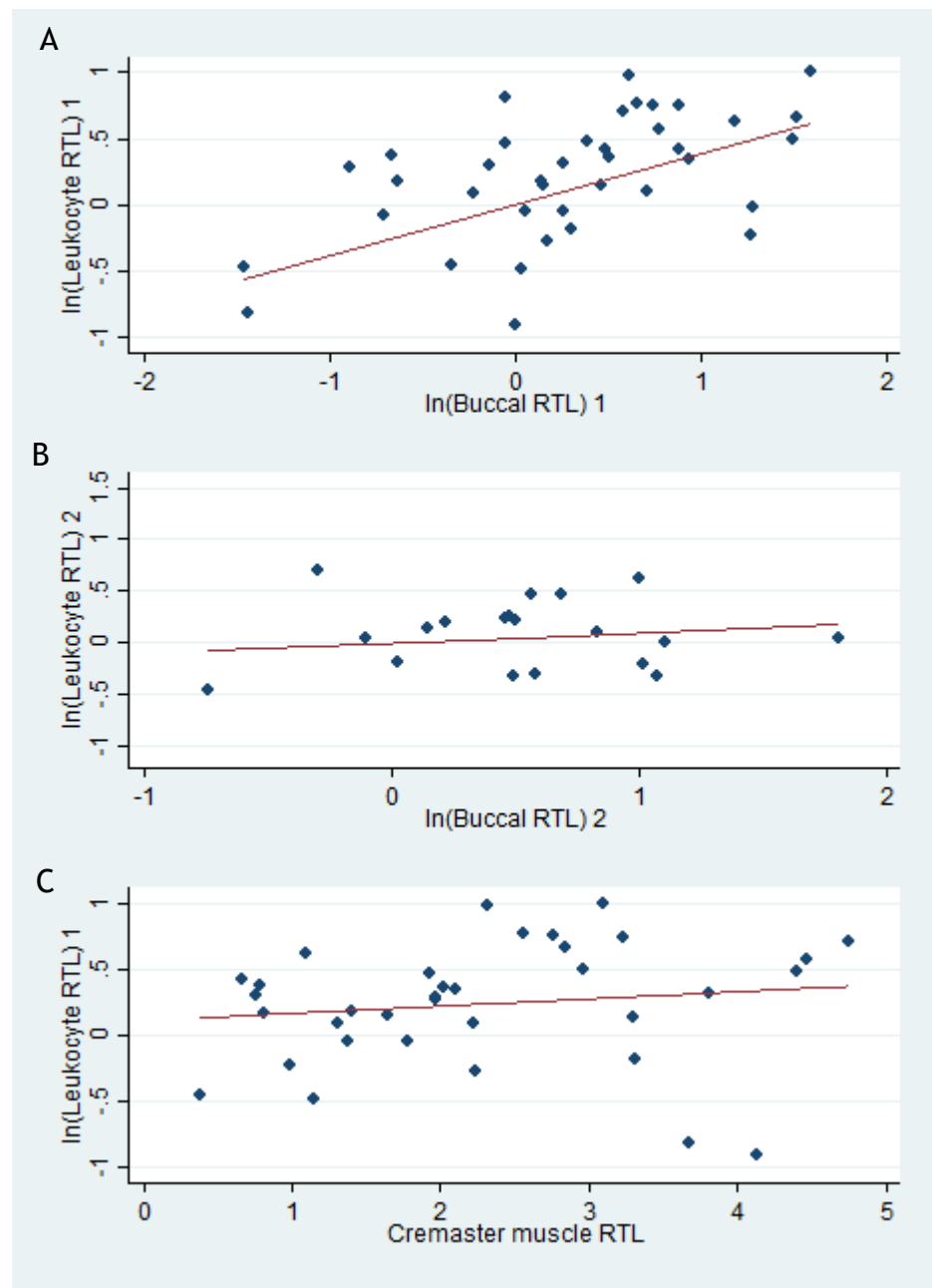


Figure 5.3 Scatterplots of relative telomere lengths in pairs of tissues

Scatterplots with regression lines of natural log of leukocyte RTL and natural log of buccal RTL in individuals at time point 1 (A) ($p < 0.001$); natural log of leukocyte RTL and natural log of buccal RTL in individuals at time point 2 (B) ($p = 0.87$); natural log of leukocyte RTL in individuals at time point 1 and cremaster muscle RTL (C) ($p = 0.41$)

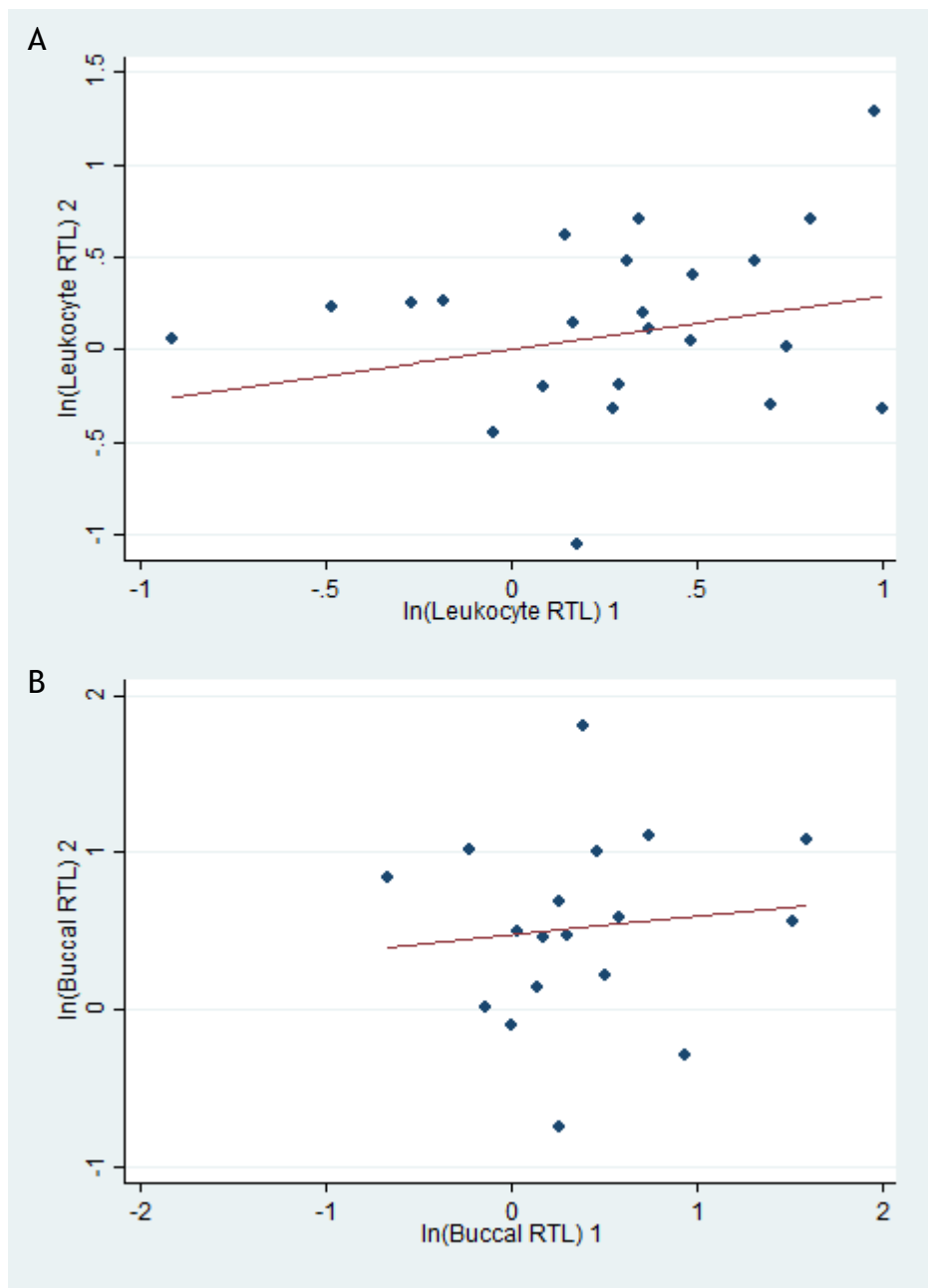


Figure 5.4 Relative telomere lengths within dogs at both time points

Scatterplots with regression line of natural log of leukocyte RTL in individuals at both time points (A) ($p=0.36$) and natural log of buccal RTL at both time points (B) ($p=0.67$).

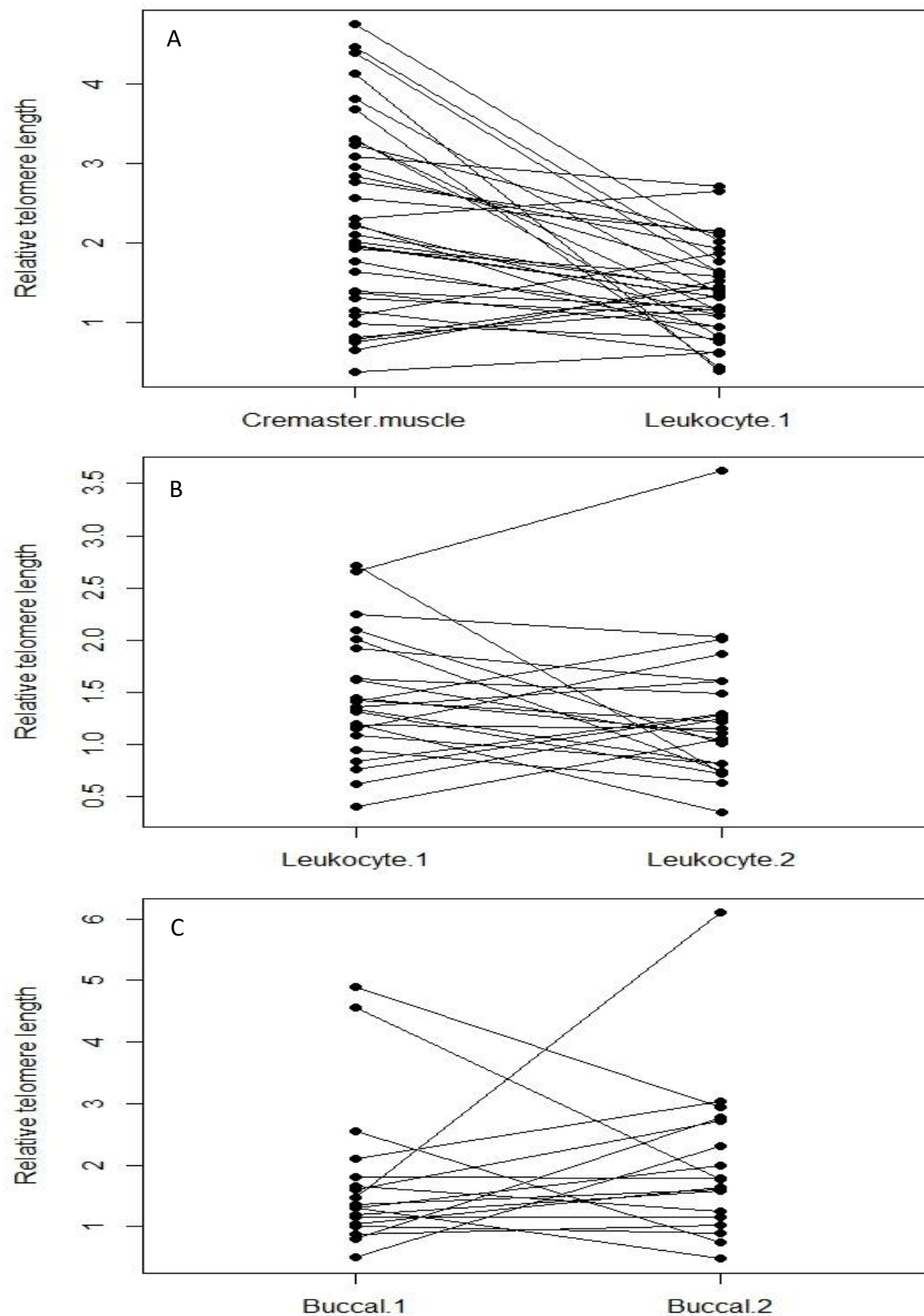


Figure 5.5 Ladder plots of pairs of samples within individuals

Ladder plots displaying pairwise values within individuals of cremaster muscle RTL and leukocyte RTL at time point 1 (A); leukocyte RTL at time points 1 and 2 (B); buccal RTL at time points 1 and 2 (C)

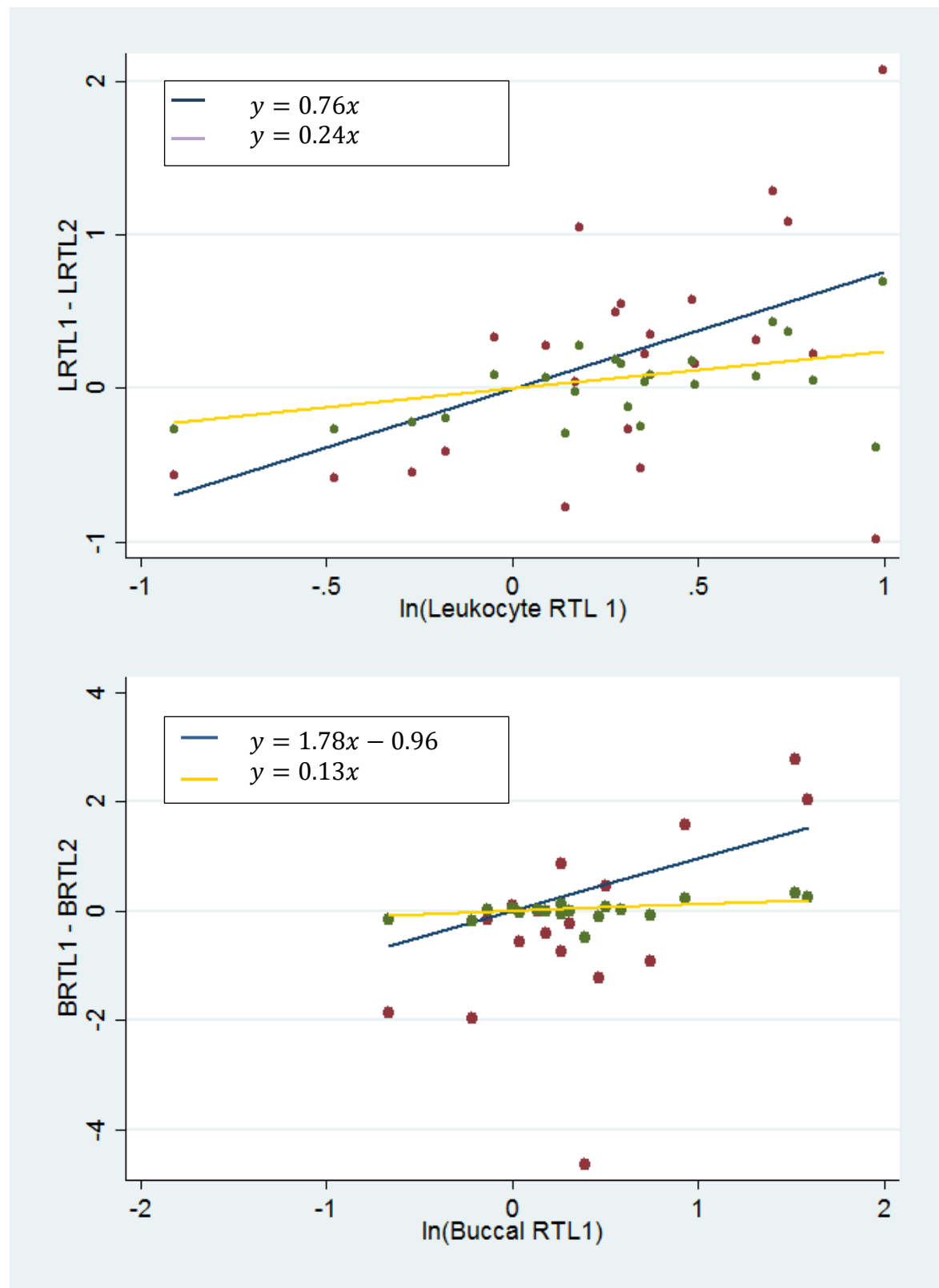


Figure 5.6 Scatterplots of the change in telomere length by the baseline telomere length

Scatterplots of the change in LRTL and BRTL between the two times point and baseline LRTL (A) and baseline BRTL (B). The red points with blue regression lines correspond to the original data, and the green points with the gold lines show the data corrected for the regression to the mean effect

5.3.4 Age

An aim of this chapter was to investigate which tissue or tissue combination out of those available was the most reflective of the age of the dogs. After log transforming age to improve model residuals, age was linearly regressed to each tissue or tissue combination. Combinations with CMRTL, ERTL or VDRTL using either leukocyte or buccal telomere lengths were used to check which of these pairs of telomere lengths could potentially be a biomarker for age. Neither of the leukocyte telomere measurement sets on their own were significantly related to age (LRTL1, $F=0.73_{(1,38)}$, $R^2=0.02$, $p=0.40$; LRTL2, $F=2.02_{(1,22)}$, $R^2=0.08$, $p=0.17$, Figures 5.7A&B). Buccal RTLs at time point 1 were not significantly regressed to age ($F=0.05_{(1,37)}$, $R^2=0.001$, $p=0.82$, Figure 5.7C) however, at time point 2, buccal RTLs significantly increased with age ($F=9.37_{(1,18)}$, $R^2=0.34$, $p=0.007$, Figure 5.7D). Although, when any of a number of variables including, for example, weight, or height, were included in the model the relationship between age and BRTL2 was no longer significant. There was no significant relationship between the change in leukocyte RTL or buccal RTL between the two time points and age (LRTL1-LRTL2, $p=0.15$; BRTL1-BRTL2, $p=0.71$) (Figure 5.8). Cremaster muscle RTLs, vas deferens RTLs and epididymis RTLs did not significantly change with age ($p=0.36$, $p=0.15$ and $p=0.32$, respectively; Figure 5.9).

Next, differences between pairs of tissue types were considered as potential age markers. The differences between LRTL1 and CMRTL, LRTL1 and ERTL, and LRTL1 and VDRTL were not significantly related to dog age ($p=0.36$, $p=0.19$ and $p=0.23$, respectively; Figure 5.10). Likewise, the difference between LRTL2 and VDRTL was not significantly associated with dog age ($p=0.28$). However, the linear models of dog age to the differences between LRTL2 and CMRTL ($F=16.94_{(1,21)}$, $R^2=0.45$, $p=0.0005$) and LRTL2 and ERTL ($F=5.37_{(1,20)}$, $R^2=0.21$, $p=0.03$) were statistically significant (Figure 5.11). The differences between BRTL1 and CMRTL, BRTL1 and ERTL, BRTL1 and VDRTL, BRTL2 and ERTL, and BRTL2 and VDRTL were not significantly associated with dog age ($p>0.1$ for all). As models with better fit were being found using LRTL with other tissues than buccal

telomere length with other tissues, buccal telomere length was only used as a single tissue moving forward.

The rate of change between tissues and LRTL1 by age were also examined. The linear model of age to the change between CMRTL and LRTL1 per month was statistically significant ($AIC=-35.48$, $p=0.02$), with older dogs having a slower rate of change (Figure 5.12A). In addition, the change between ERTL and LRTL1 per month of life also had a significant relationship with dog age ($AIC=-51.93$, $p=0.01$) (Figure 5.12B). This was not the case, however, when vas deferens was used as the comparative tissue ($AIC=-35.04$, $p=0.10$). None of the age to rates of change linear models for time point 2 were statistically significant: CMRTL minus LRTL2 per month ($p=0.13$), VDRTL minus LRTL2 per month ($p=0.22$) and ERTL minus LRTL2 per month ($p=0.28$).

To see if dog age as a proportion of their life expectancy (LE) was related to telomere length, age was divided by breed life expectancy and tested for association with telomere length. Age divided by LE was log transformed to improve model residuals. There was a positive trend between LRTL1 and life expectancy corrected age ($F=3.54_{(1,28)}$, $R^2=0.11$, $p=0.07$, Figure 5.13A). The linear model of LE adjusted age to BRTL1 was statistically significant, with a negative relationship observed ($F=6.13_{(1,28)}$, $R^2=0.18$, $p=0.02$, Figure 5.13B). The linear models of age to LRTL2 and BRTL2 were not statistically significant ($p=0.35$, $p=0.09$ respectively). Likewise, there was no significant relationship between CMRTL and LE adjusted age ($p=0.82$). The linear models of LE adjusted age to RTL ($F=27.37_{(1,24)}$, $R^2=0.53$, $p<0.001$, Figure 5.13C) and age to VDRTL ($F=20.08_{(1,24)}$, $R^2=0.46$, $p<0.001$, Figure 5.13D) were both statistically significant. The linear model of LE adjusted age to the change in LRTL between time points 1 and 2 was not statistically significant ($F<0.001_{(1,14)}$, $R^2<0.001$, $p=0.99$). The change in buccal RTL was also not related to LE adjusted age ($F=0.11_{(1,9)}$, $R^2=0.01$, $p=0.75$).

Interestingly, the differences between LRTL1 and CMRTL, and LRTL2 and CMRTL significantly decreased with LE adjusted age ($AIC=80.03$, $p=0.003$ and $F=5.95_{(1,13)}$, $R^2=0.31$, $p=0.03$; Figure 5.14A/B). The regression lines in these plots have a positive beta coefficient as the difference between the tissues approaches zero with increasing age. In addition, the rate of change per month between CMRTL and LRTL1, and CMRTL and LRTL2 decreased with increasing LE adjusted age ($AIC=-32.0$, $p=0.002$ and $F=9.98_{(1,13)}$, $R^2=0.43$, $p=0.008$; Figure 5.14C/D). Likewise, the differences between LRTL1 and VDRTL, and LRTL2 and VDRTL decreased with LE adjusted age ($F=12.52_{(1,24)}$, $R^2=0.34$, $p=0.002$ and $F=7.80_{(1,13)}$, $R^2=0.38$, $p=0.02$, respectively. Figure 5.17A/B). In addition, as with CMRTL, the rate of change between VDRTL and both LRTL1 and LRTL2 decreased with LE adjusted age ($AIC=-27.03$, $p=0.001$ and $AIC=-31.81$, $p=0.02$, respectively. Figure 5.17C/D). These findings were replicated in the differences between LRTL and ERTL, however at time point 2 there were non-statistically significant trends (LRTL1 minus ERTL: $F=11.4_{(1,24)}$, $R^2=0.32$, $p=0.002$; LRTL2 minus ERTL: $F=3.80_{(1,13)}$, $R^2=0.23$, $p=0.07$; ERTL minus LRTL1 per month $AIC=-51.31$, $p<0.001$; ERTL minus LRTL2 per month $F=4.44_{(1,13)}$, $R^2=0.25$, $p=0.06$. Figure 5.16).

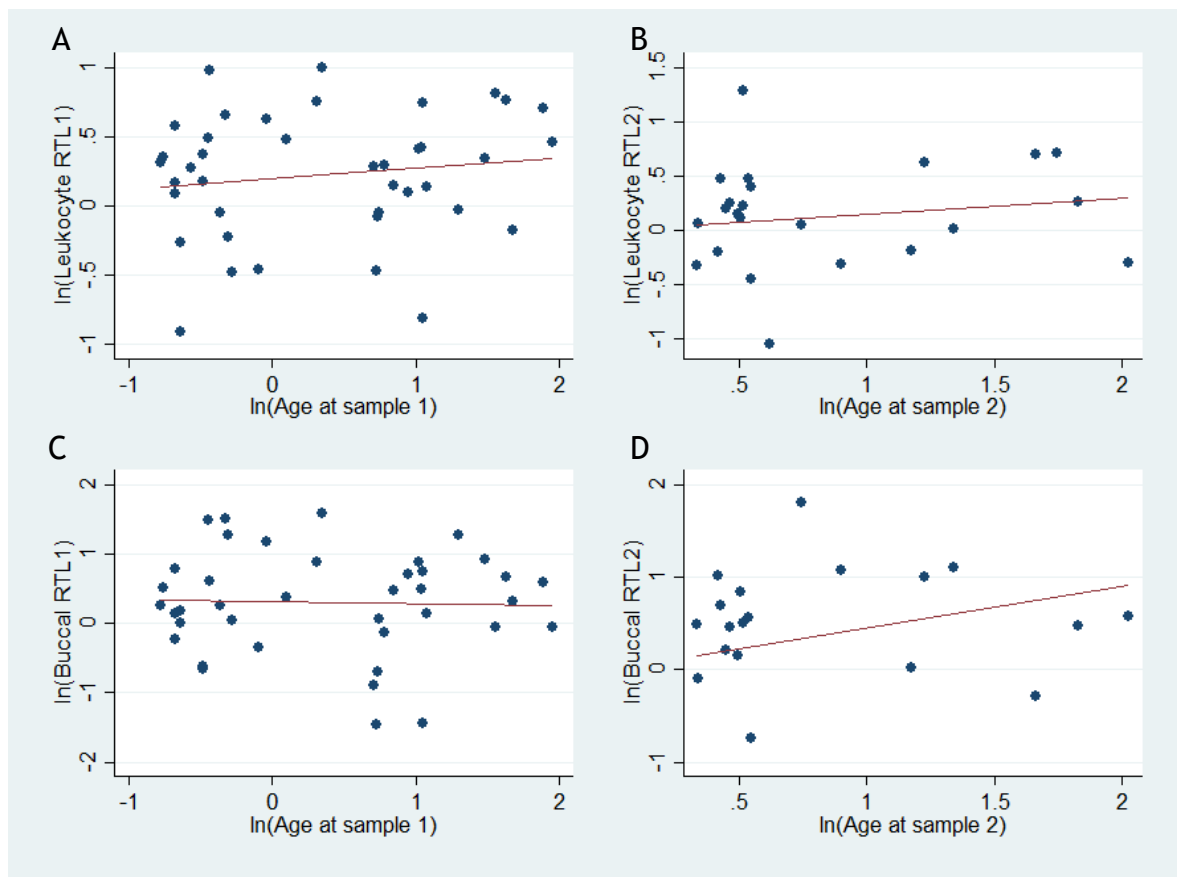


Figure 5.7 Scatterplots of tissue telomere lengths by age

Scatterplots with regression line of natural log of leukocyte RTL and natural log of age at time point 1(A) ($R^2=0.02$, $p=0.40$); natural log of leukocyte RTL and natural log of age at time point 2 (B) ($R^2=0.08$, $p=0.17$); natural log of buccal RTL and natural log of age at time point 1 (C) ($R^2=0.001$, $p=0.82$); natural log of buccal RTL and natural log of age at time point 2(D) ($R^2=0.34$, $p=0.007$).

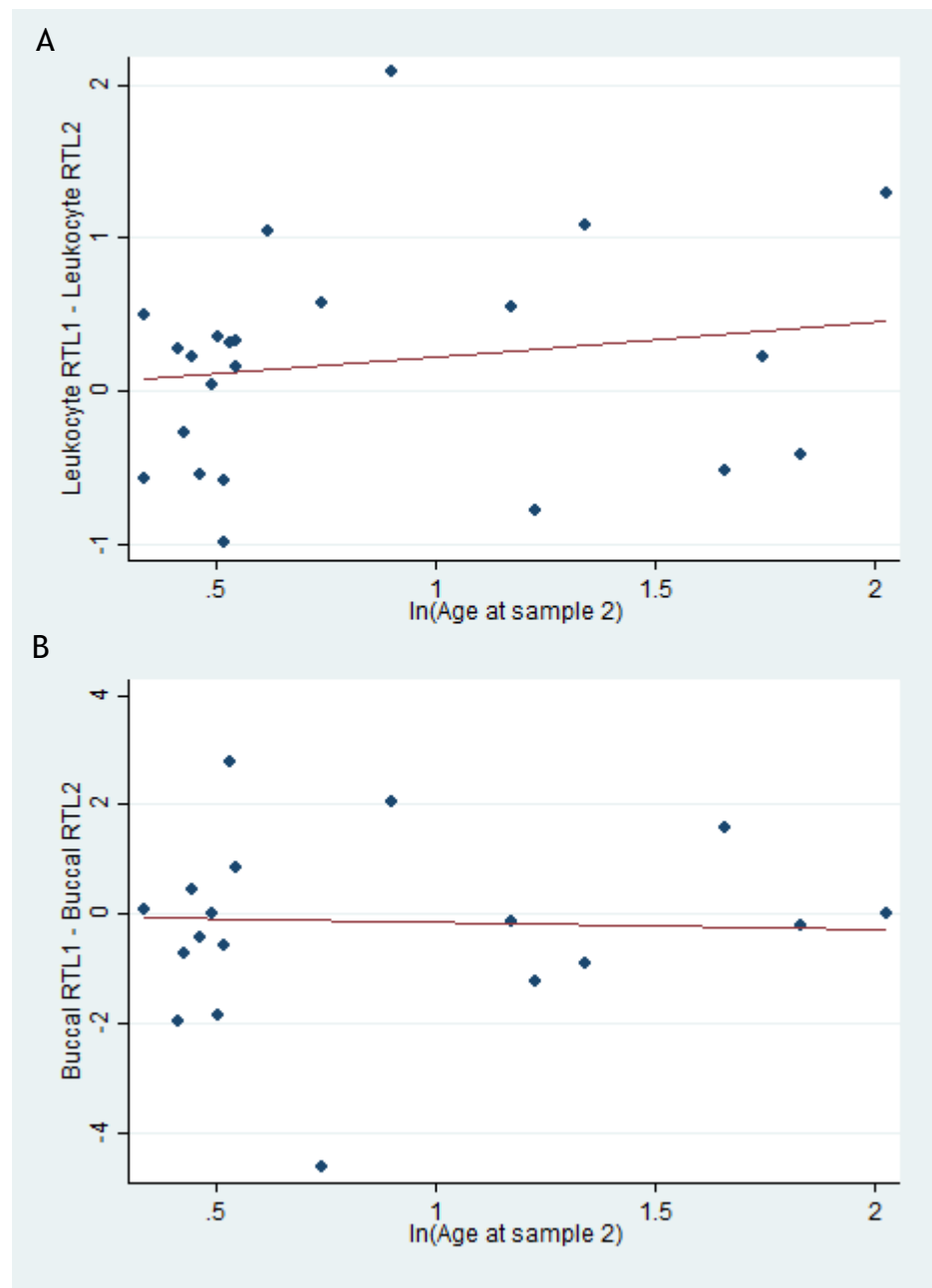


Figure 5.8 Scatterplots of change in telomere length between the time points by age

Scatterplots with regression line of leukocyte RTL 1 minus leukocyte RTL 2 and natural log of age at time point 2 (A) ($R^2=0.09$, $p=0.15$); change in buccal RTL between time points 1 and 2 and natural log of age at time point 2 (B) ($R^2=0.008$, $p=0.71$)

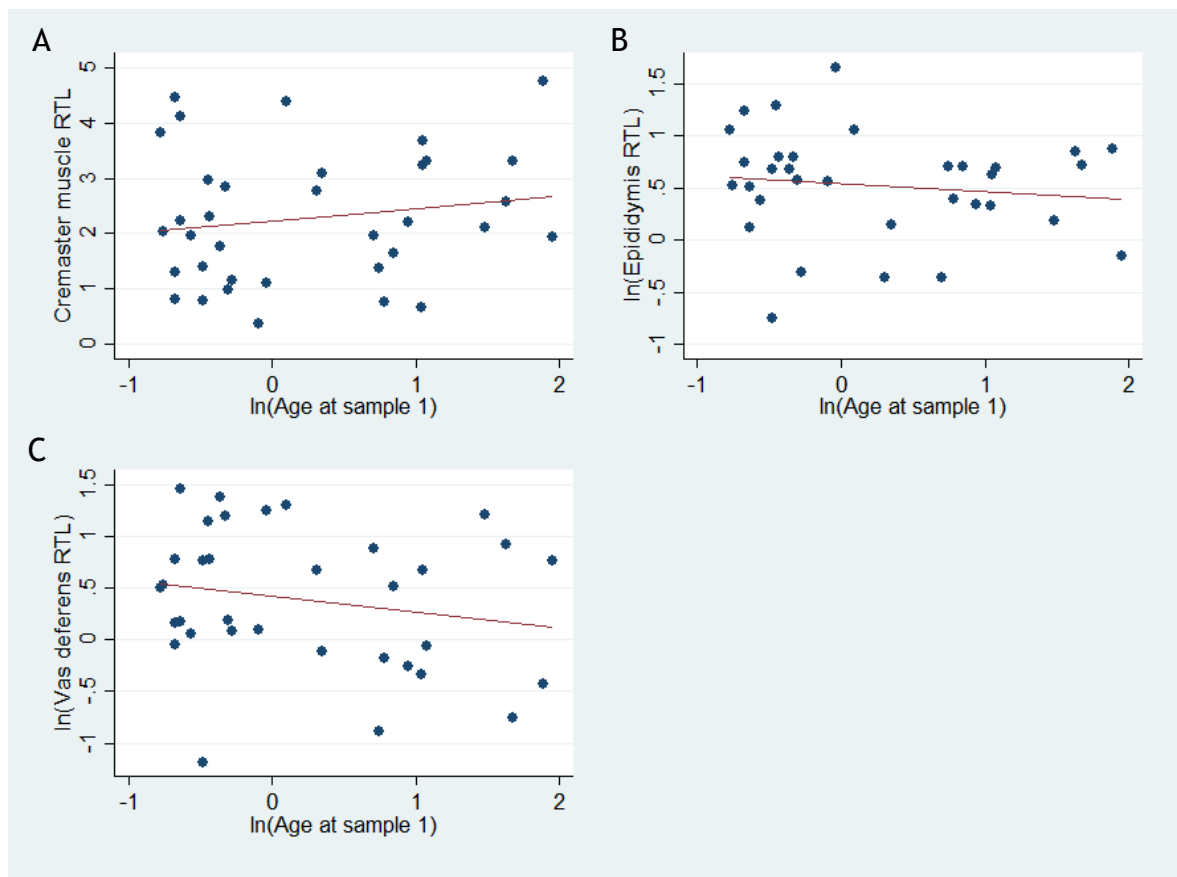


Figure 5.9 Scatterplots of CMRTL, VDRTL and ERTL by age

Scatterplots with regression line of cremaster muscle RTL and natural log of age at time point 1 (A) ($R^2=0.03$, $p=0.36$); natural log of epididymis RTL and natural log of age at time point 1 (B) ($R^2=0.02$, $p=0.49$) and natural log of vas deferens RTL and natural log of age at time point 1 (C) ($R^2=0.03$, $p=0.32$).

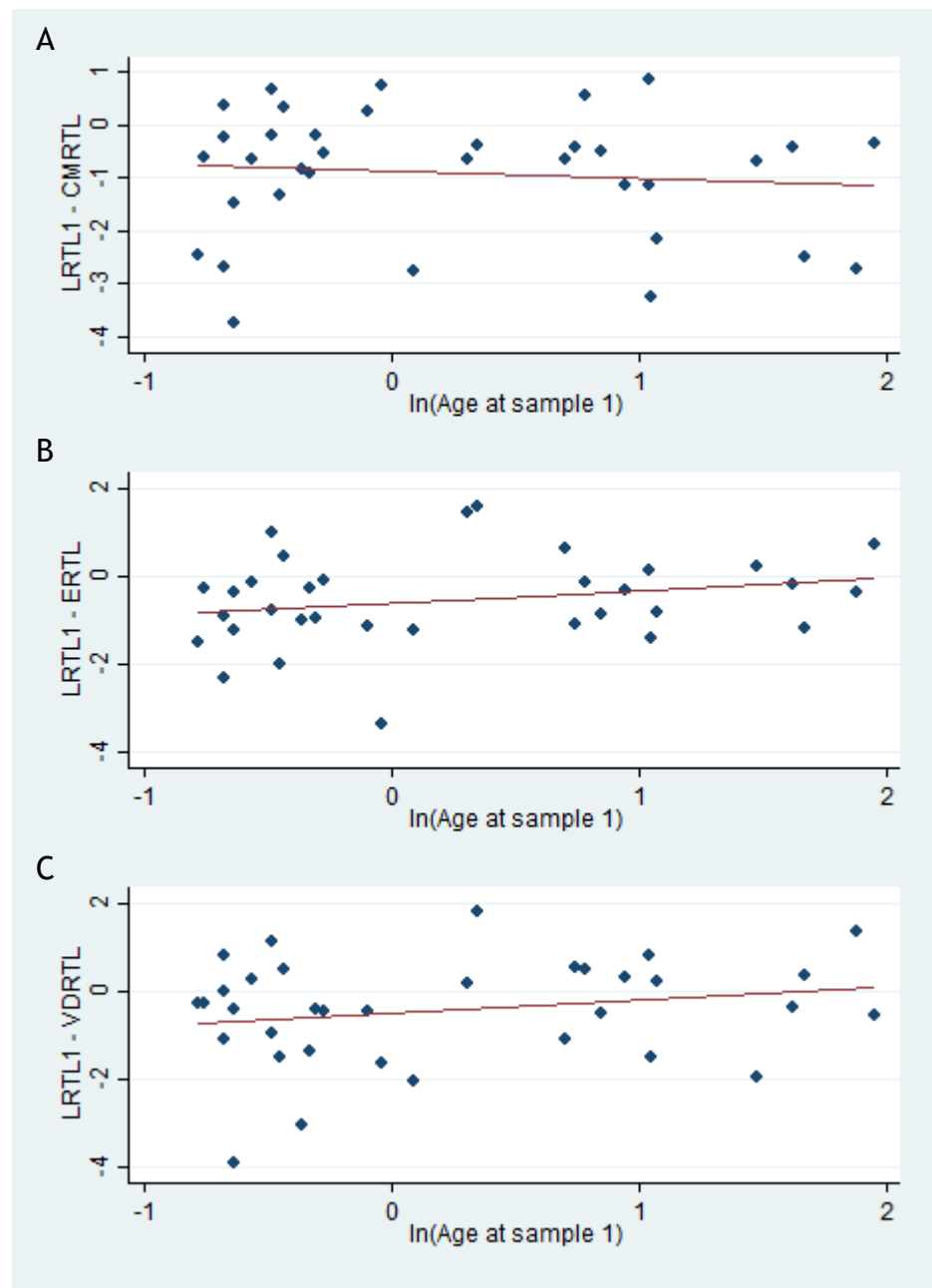


Figure 5.10 Difference between tissue telomere lengths and age 1

Scatterplots with regression line of difference in leukocyte RTL and cremaster muscle RTL and natural log of age at time point 1 (A) (AIC=116.59, $p=0.36$); difference between leukocyte RTL and epididymis RTL and natural log of age at time point 1 (B) ($R^2=0.06$, $p=0.19$); difference between leukocyte RTL and vas deferens RTL and natural log of age at time point 1 (C) ($R^2=0.04$, $p=0.23$)

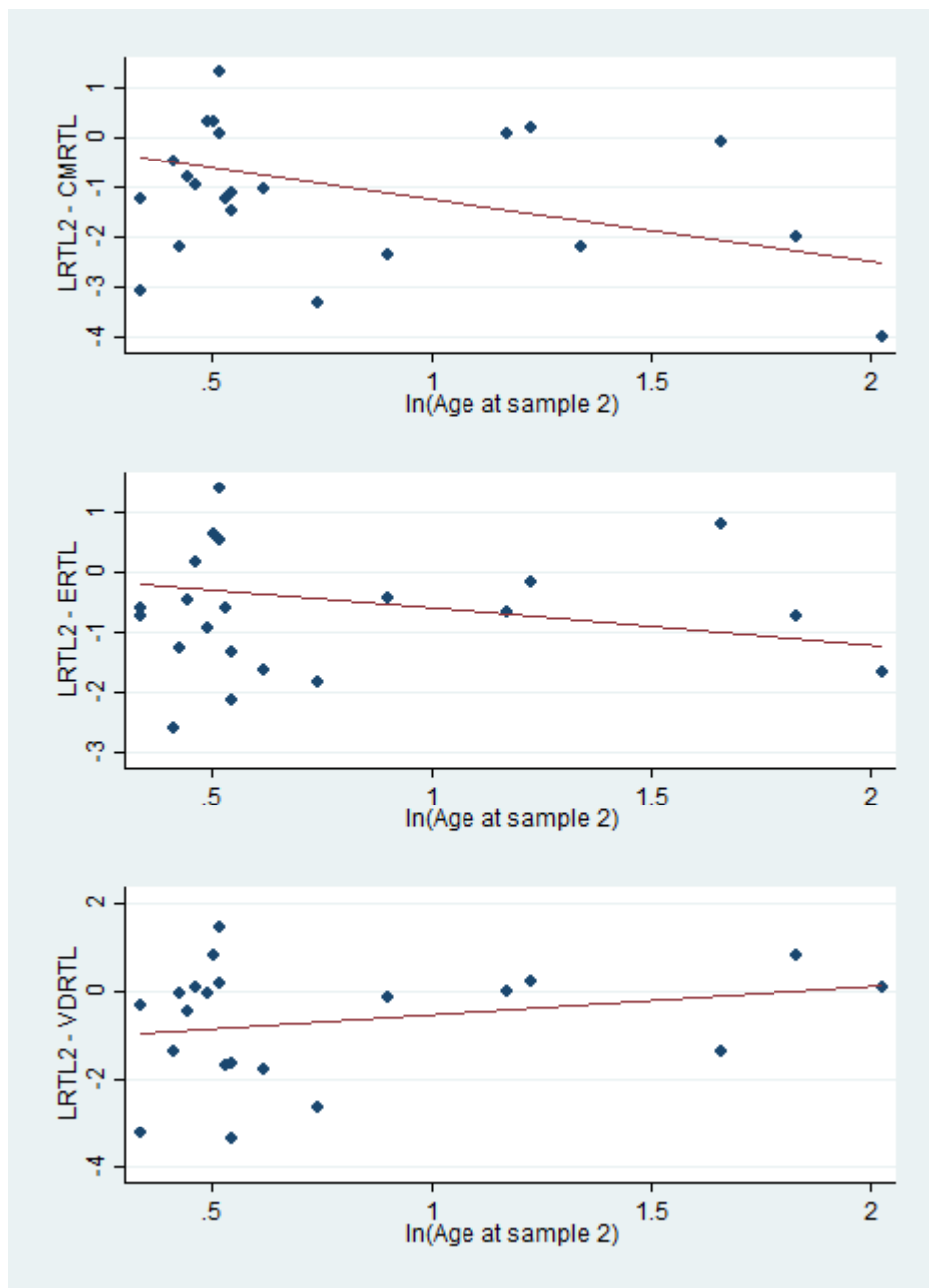


Figure 5.11 Differences between tissue telomere lengths and age 2

Scatterplots with regression line of difference in leukocyte RTL and cremaster muscle RTL and natural log of age at time point 2 (A) ($R^2=0.45$, $p<0.001$); difference between leukocyte RTL and epididymis RTL and natural log of age at time point 2 (B) ($R^2=0.21$, $p=0.03$); difference between leukocyte RTL and vas deferens RTL and natural log of age at time point 2 (C) ($R^2=0.06$, $p=0.28$)

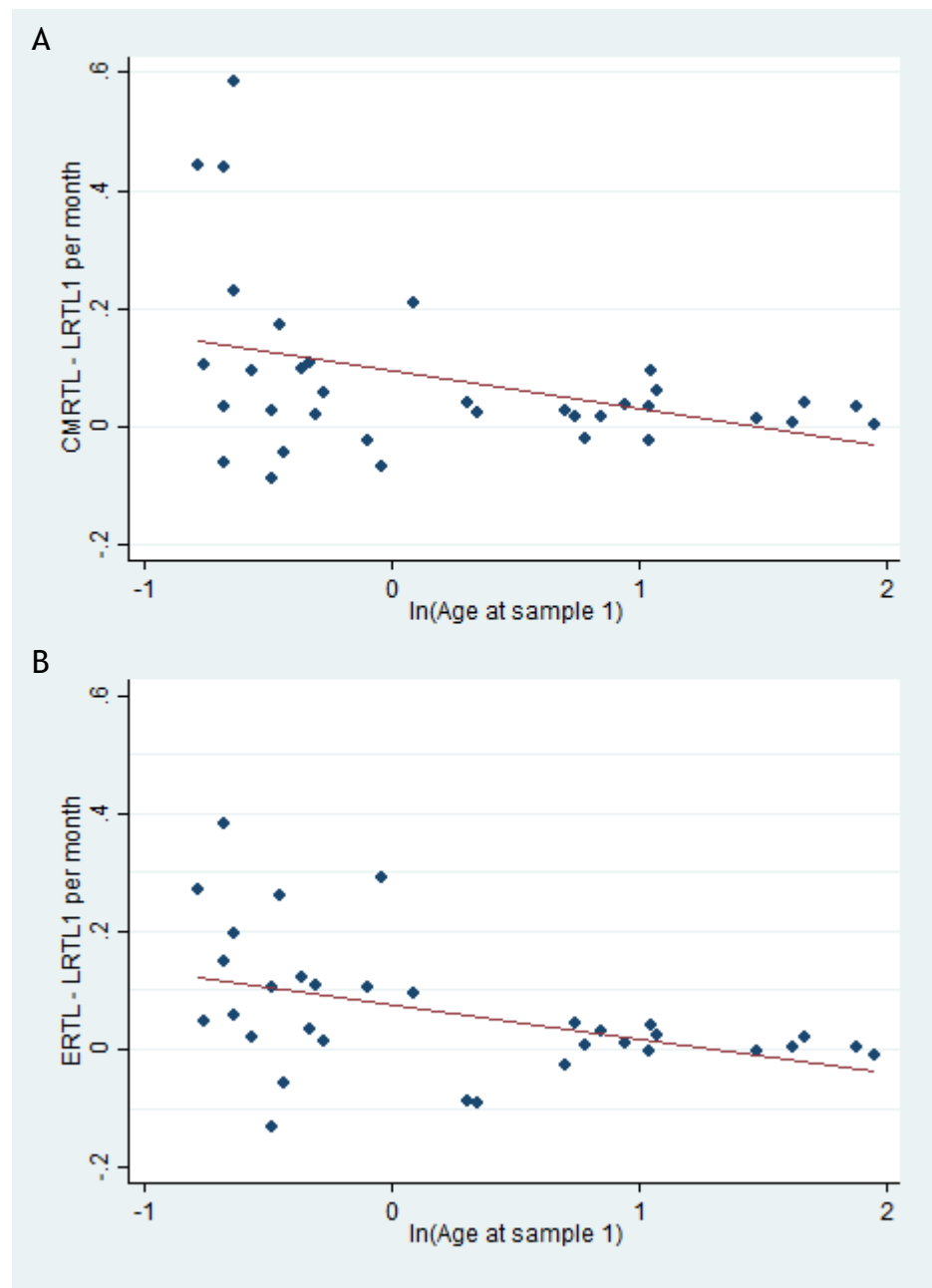


Figure 5.12 Change between telomere lengths over life by age 1

Scatterplots with regression line of rate of change between leukocyte RTL and cremaster muscle RTL per month and natural log of age at time point 1 (A) (AIC=-35.48, $p=0.02$); and rate of change between leukocyte RTL and epididymis RTL and natural log of age at time point 1 (B) (AIC=-51.93, $p=0.01$)

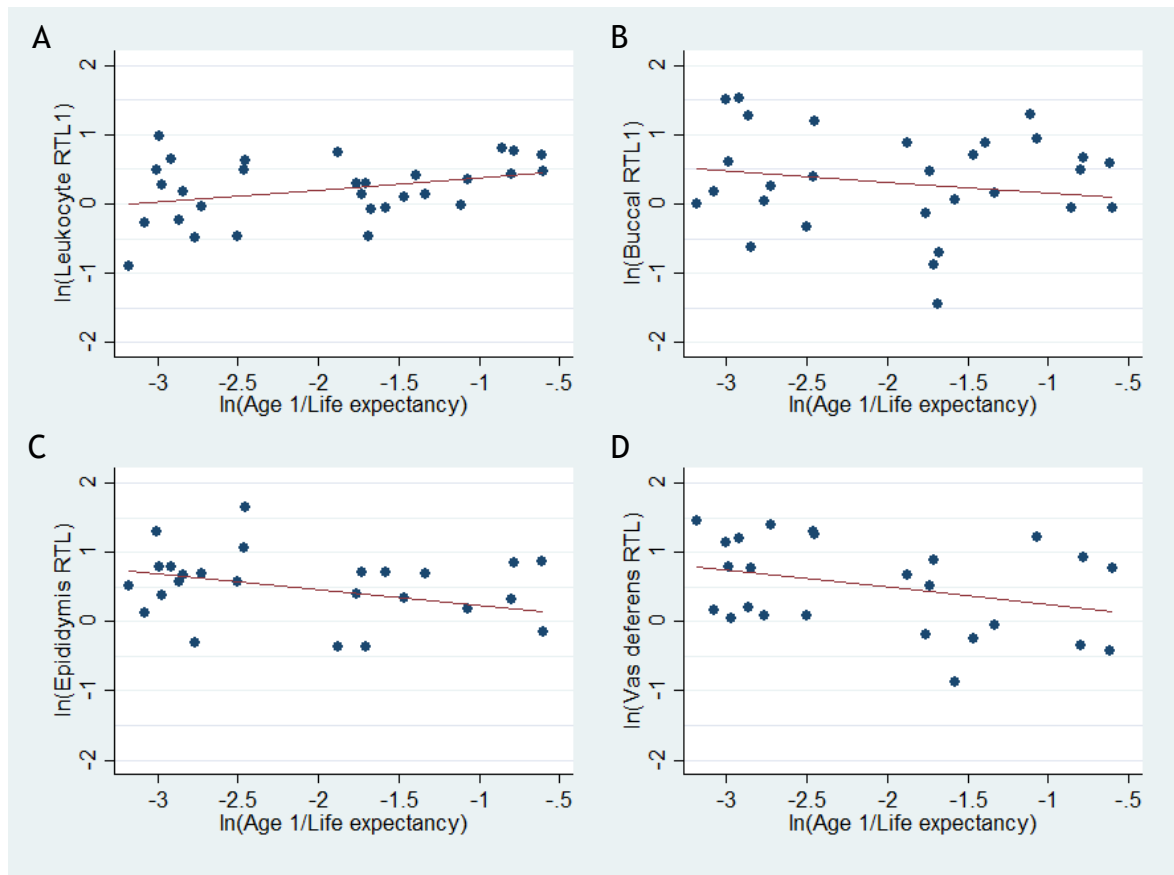


Figure 5.13 Telomere lengths by life expectancy adjusted age

Scatterplots with regression line of natural log of leukocyte RTL and natural log of age divided by breed life expectancy at time point 1 (A) ($R^2=0.11$, $p=0.07$); natural log of buccal RTL and natural log of age divided by breed life expectancy at time point 1 (B) ($R^2=0.18$, $p=0.02$); natural log of epididymis RTL and natural log of age divided by breed life expectancy (C) ($R^2=0.53$, $p<0.001$); natural log of vas deferens RTL and natural log of age divided by breed life expectancy (D) ($R^2=0.46$, $p<0.001$).

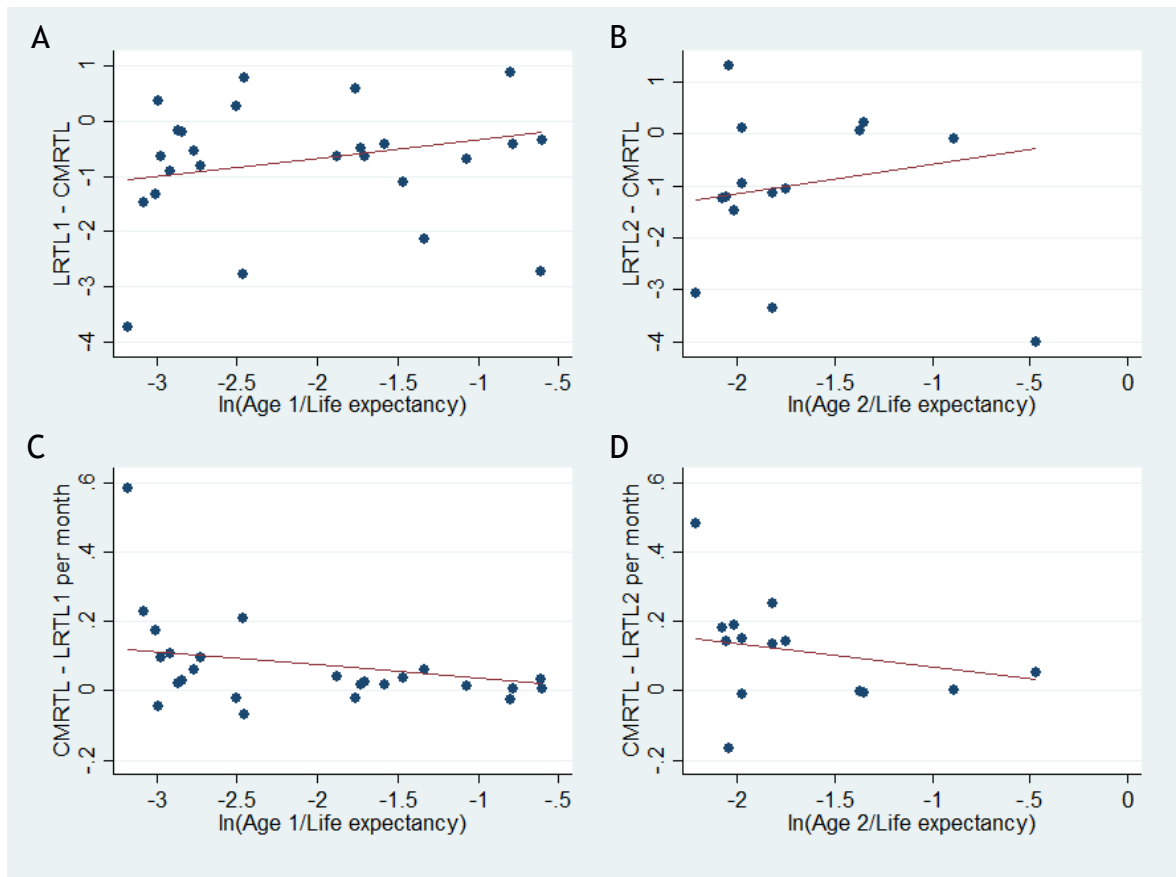


Figure 5.14 Differences between tissue telomere lengths by life expectancy adjusted age 1

Scatterplots with regression line of difference in leukocyte RTL and cremaster muscle RTL and natural log of age divided by breed life expectancy at time point 1 (A) (AIC=80.03, $p=0.003$); difference between leukocyte RTL and cremaster muscle RTL and natural log of age divided by breed life expectancy at time point 2 (B) ($R^2=0.31$, $p=0.03$); rate of change between leukocyte RTL and cremaster muscle RTL per month and natural log of age divided by breed life expectancy at time point 1 (C) (AIC=-32.0, $p=0.002$); and rate of change between leukocyte RTL and cremaster muscle RTL and natural log of age divided by breed life expectancy at time point 2 (D) ($R^2=0.43$, $p=0.008$)

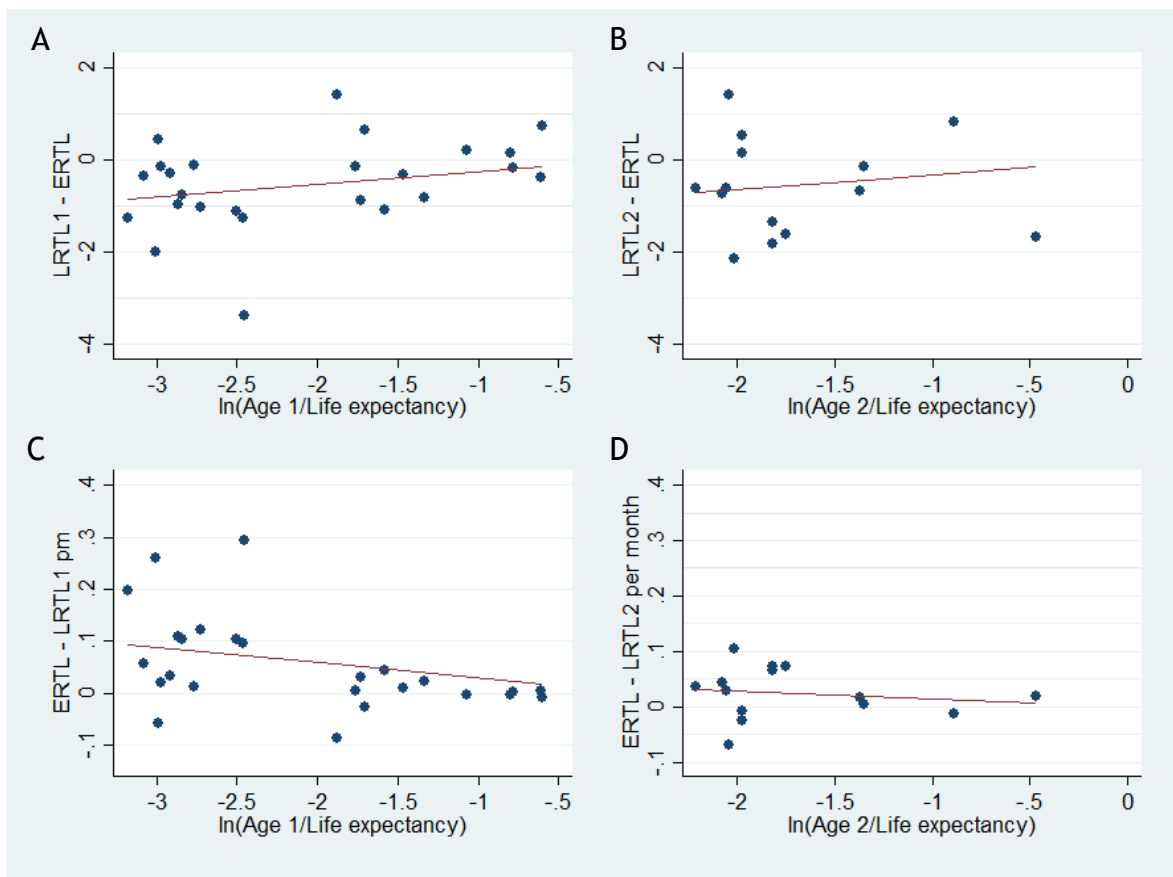


Figure 5.15 Differences between tissue telomere lengths by life expectancy adjusted age 2

Scatterplots with regression line of difference in leukocyte RTL and epididymis RTL and natural log of age divided by breed life expectancy at time point 1 (A) ($R^2=0.32$, $p=0.002$); difference between leukocyte RTL and epididymis RTL and natural log of age divided by breed life expectancy at time point 2 (B) ($R^2=0.23$, $p=0.07$); rate of change between leukocyte RTL and epididymis RTL per month and natural log of age divided by breed life expectancy at time point 1 (C) ($AIC=-51.31$, $p<0.001$); and rate of change between leukocyte RTL and epididymis RTL and natural log of age divided by breed life expectancy at time point 2 (D) ($R^2=0.25$, $p=0.06$)

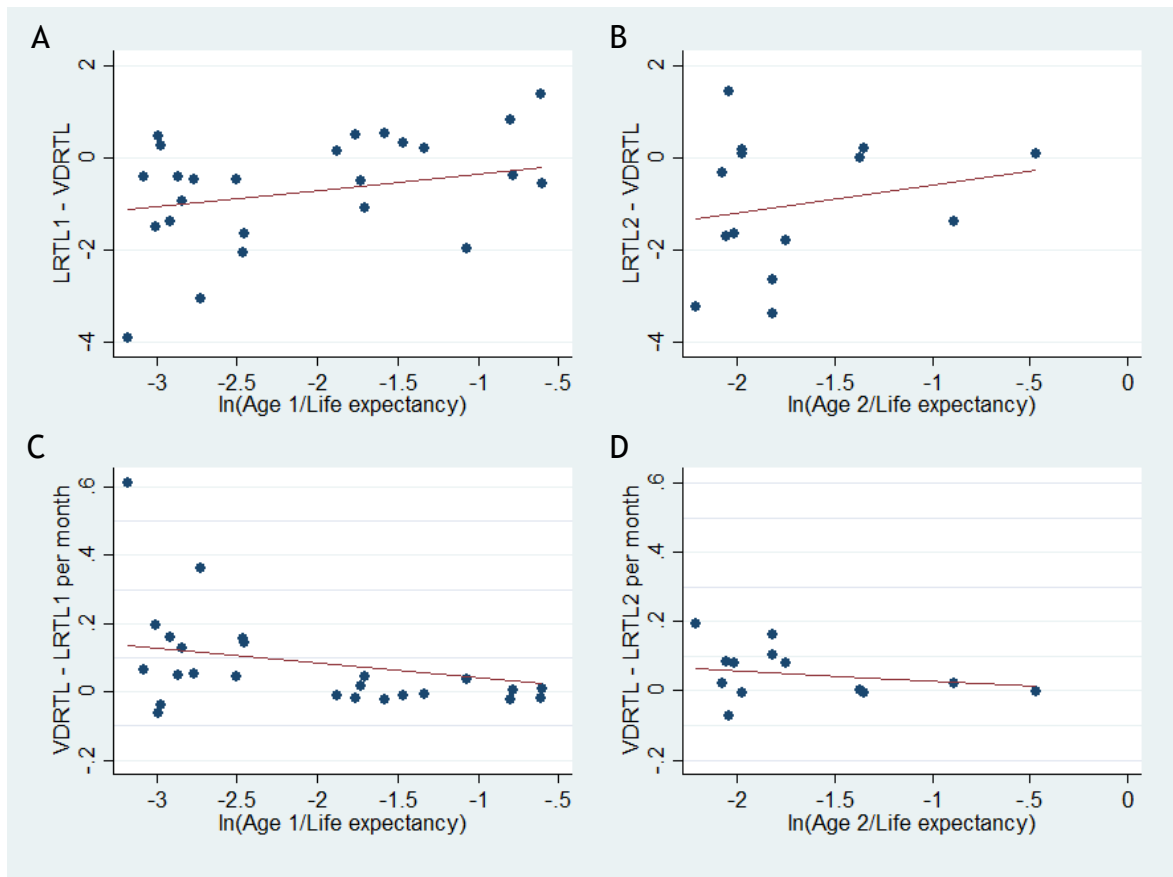


Figure 5.16 Differences between tissue telomere lengths by life expectancy adjusted age 3

Scatterplots with regression line of difference in leukocyte RTL and vas deferens RTL and natural log of age divided by breed life expectancy at time point 1 (A) ($R^2=0.34$, $p=0.002$); difference between leukocyte RTL and vas deferens RTL and natural log of age divided by breed life expectancy at time point 2 (B) ($R^2=0.38$, $p=0.02$); rate of change between leukocyte RTL and vas deferens RTL per month and natural log of age divided by breed life expectancy at time point 1 (C) ($AIC=-27.03$, $p=0.001$); and rate of change between leukocyte RTL and vas deferens RTL and natural log of age divided by breed life expectancy at time point 2 (D) ($AIC=-31.81$, $p=0.02$)

5.3.5 Nicotine

Hair nicotine and cotinine concentrations (HNC and HCC) were used as objective measures of ETS exposure. These markers were examined against telomere length in each tissue to see if there was an association present. HNC was log transformed and the square root of HCC taken as per Chapter 3 to improve model residuals.

A significant linear model was found between LRTL1 and HNC1 ($F=6.25_{(1,38)}$, $R^2=0.14$, $p=0.02$), with higher nicotine exposure being associated with shorter LRTL. There was also a negative relationship between leukocyte RTL and HNC at time point 2, however the linear model was not statistically significant ($F=4.21_{(1,22)}$, $R^2=0.16$, $p=0.05$). There was a negative, significant relationship between buccal RTL and HNC at time point 1 ($F=8.16_{(1,37)}$, $R^2=0.18$, $p=0.007$) and also at time point 2 ($F=9.62_{(1,18)}$, $R^2=0.35$, $p=0.006$) (Figure 5.17). The change in leukocyte RTL and buccal RTL between the two time points did not depend on HNC2 (LRTL1-LRTL2, $p=0.38$; BRTL1-BRTL2, $p=0.98$; Figure 5.18).

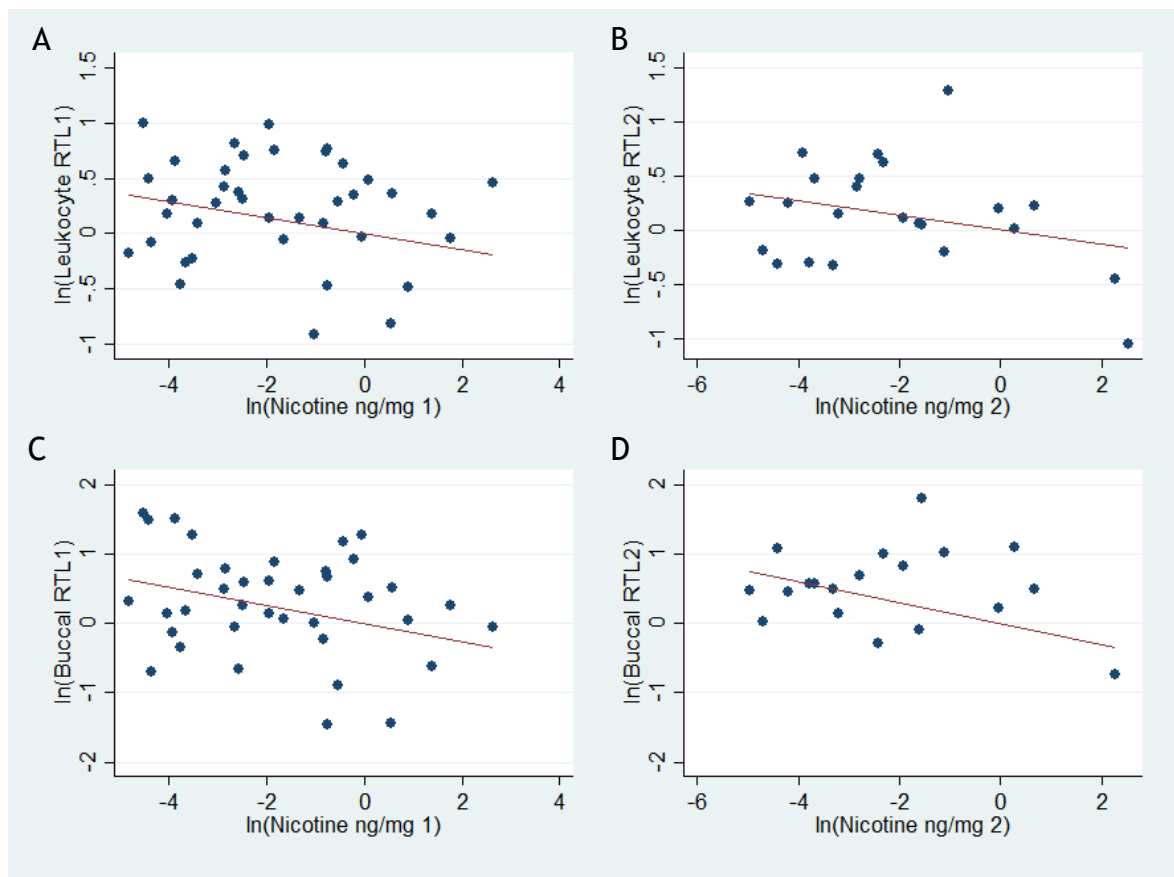


Figure 5.17 Telomere lengths by ETS biomarker concentrations 1

Scatterplots with regression line of natural log of leukocyte RTL and natural log of hair nicotine at time point 1(A) ($R^2=0.14$, $p=0.02$); natural log of leukocyte RTL and natural log of hair nicotine at time point 2 (B) ($R^2=0.16$, $p=0.05$); natural log of buccal RTL and natural log of hair nicotine at time point 1 (C) ($R^2=0.18$, $p=0.007$); and natural log of buccal RTL and natural log of hair nicotine at time point 2(D) ($R^2=0.35$, $p=0.006$)

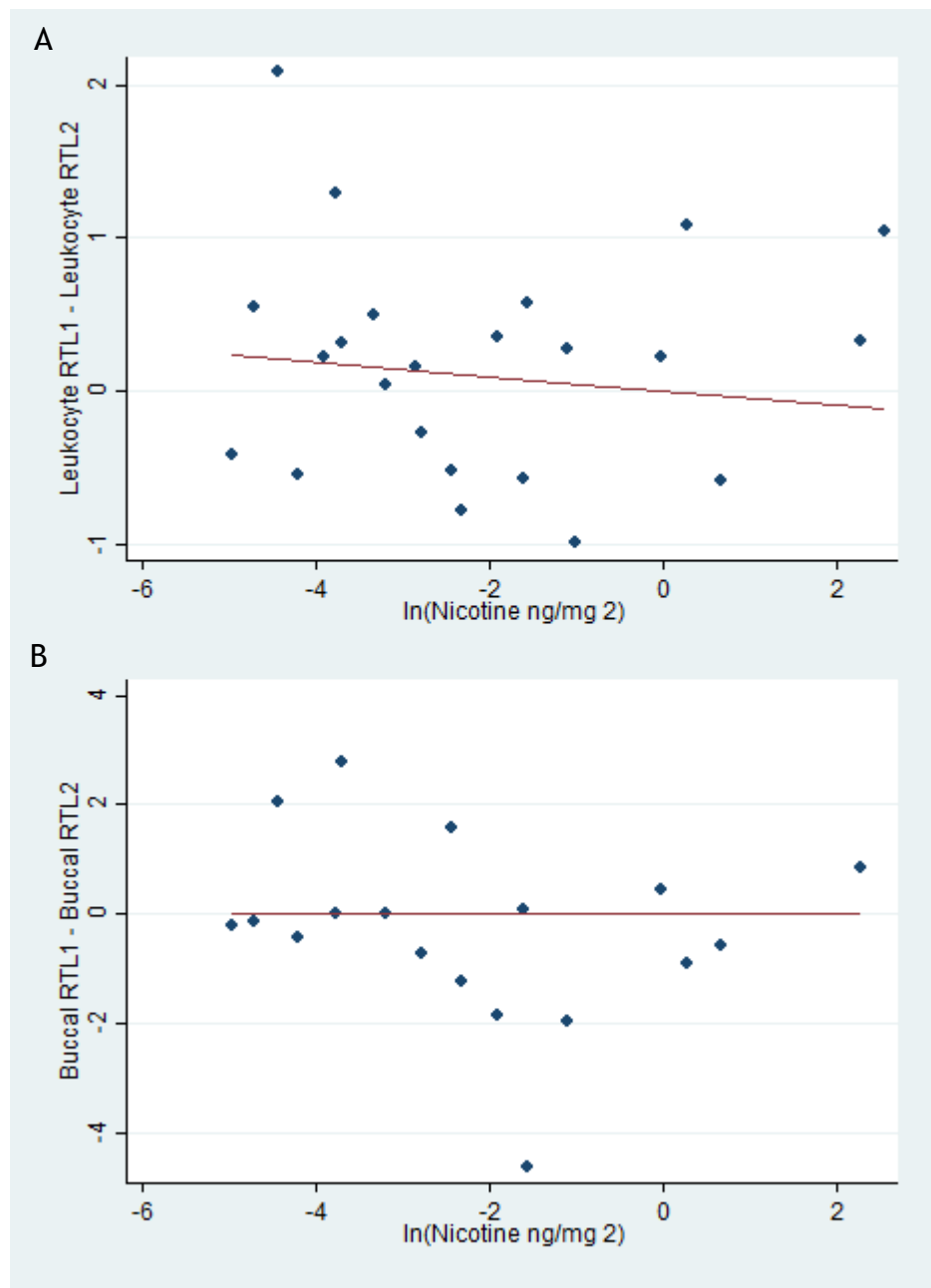


Figure 5.18 Changes in telomere lengths between study time points and HNC

Scatterplots with regression line of leukocyte RTL 1 minus leukocyte RTL 2 and natural log of hair nicotine at time point 2 (A) ($R^2=0.03$, $p=0.38$); change in buccal RTL between time points 1 and 2 and natural log of hair nicotine at time point 2 (B) ($R^2<0.001$, $p=0.98$)

Cremaster muscle RTL and epididymis RTL were not significantly related to HNC1 ($p=0.94$ and $p=0.66$, respectively). However, vas deferens RTL significantly increased with HNC1 ($F=9.92_{(1,32)}$, $R^2=0.24$, $p=0.004$) (Figure 5.19). Interestingly, when LE adjusted age was added into the linear model of HNC1 to VDRTL, both explanatory variables remained significant, with the beta coefficient for HNC1

remaining positive and the beta coefficient for LE adjusted age remaining negative ($F=16.86_{(2,23)}$, $R^2=0.59$, $p<0.001$).

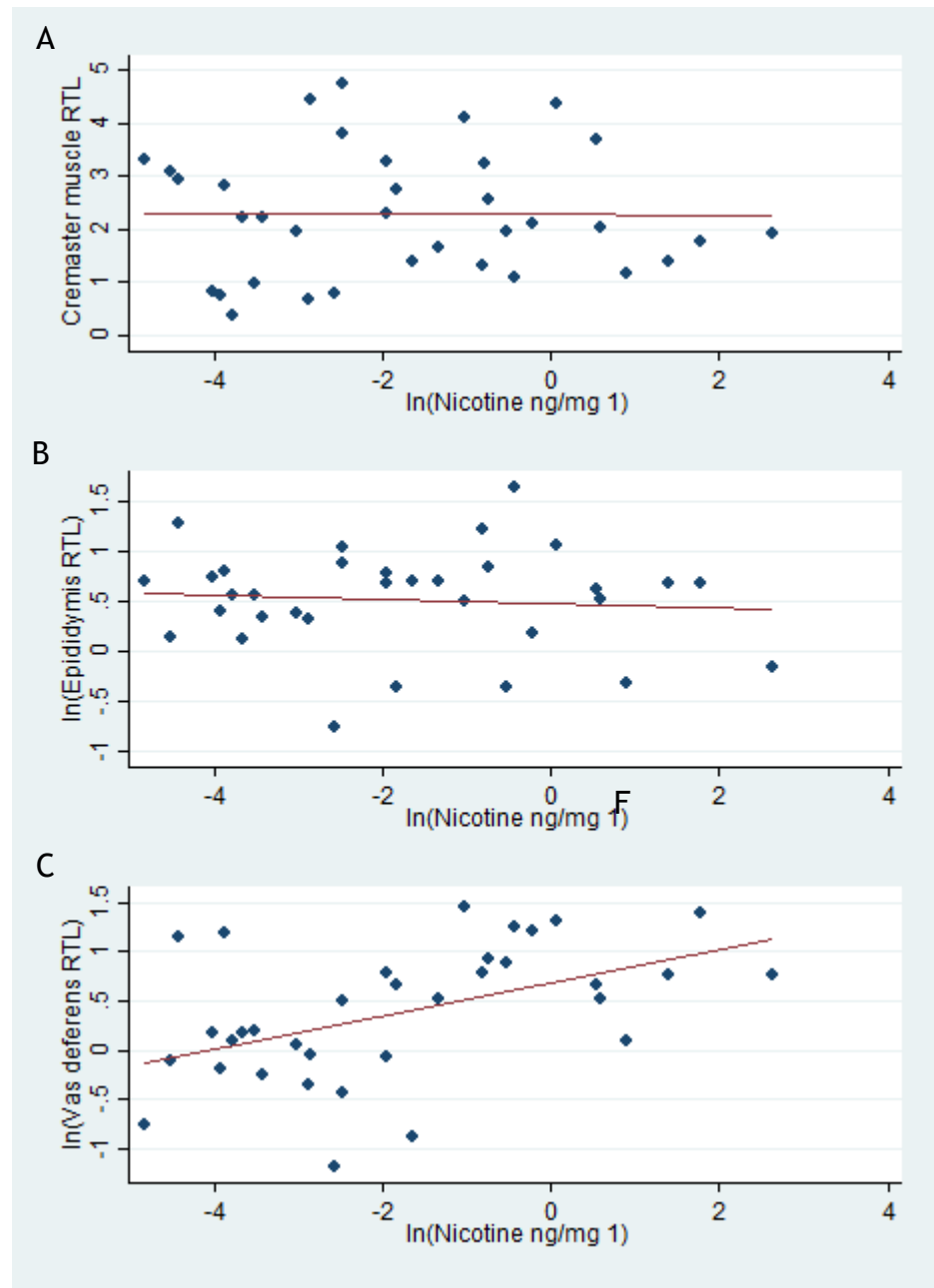


Figure 5.19 Telomere lengths by ETS biomarker concentrations 2

Scatterplots with regression line of cremaster muscle RTL and natural log of hair nicotine at time point 1 (A) ($R^2=0.0002$, $p=0.94$); natural log of epididymis RTL and natural log of hair nicotine at time point 1 (B) ($R^2=0.006$, $p=0.66$); and natural log of vas deferens RTL and natural log of hair nicotine at time point 1 (C) ($R^2=0.24$, $p=0.004$).

As well as CMRTL and ERTL as a lone variable not being significantly related to HNC1, the linear models of HNC1 to the differences between LRTL1 and CMRTL or LRTL1 and ERTL were not significant ($p=0.82$, $p=0.80$, respectively, Figure 5.20). The linear model of HNC1 to the difference between LRTL1 and VDRTL was significant ($F=10.3_{(1,32)}$, $R^2=0.24$, $p=0.003$). There were also no significant linear models between HNC2 and the differences between LRTL2 and CMRTL ($p=0.51$), and LRTL2 and ERTL ($p=0.77$). A downward, non-significant, trend existed between the difference between LRTL2 and VDRTL with HNC2 ($p=0.05$, Figure 5.20D).

The rate of change between tissues per month was also examined to see if it changed with HNC. CMRTL minus LRTL1 per month, CMRTL minus LRTL2 per month, VDRTL minus LRTL1 per month, VDRTL minus LRTL2 per month, ERTL minus LRTL1 per month and ERTL minus LRTL2 all did not have statistically significant linear models with HNC ($p=0.96$, $p=0.94$, $p=0.07$, $p=0.08$, $p=0.73$ and $p=0.48$, respectively).

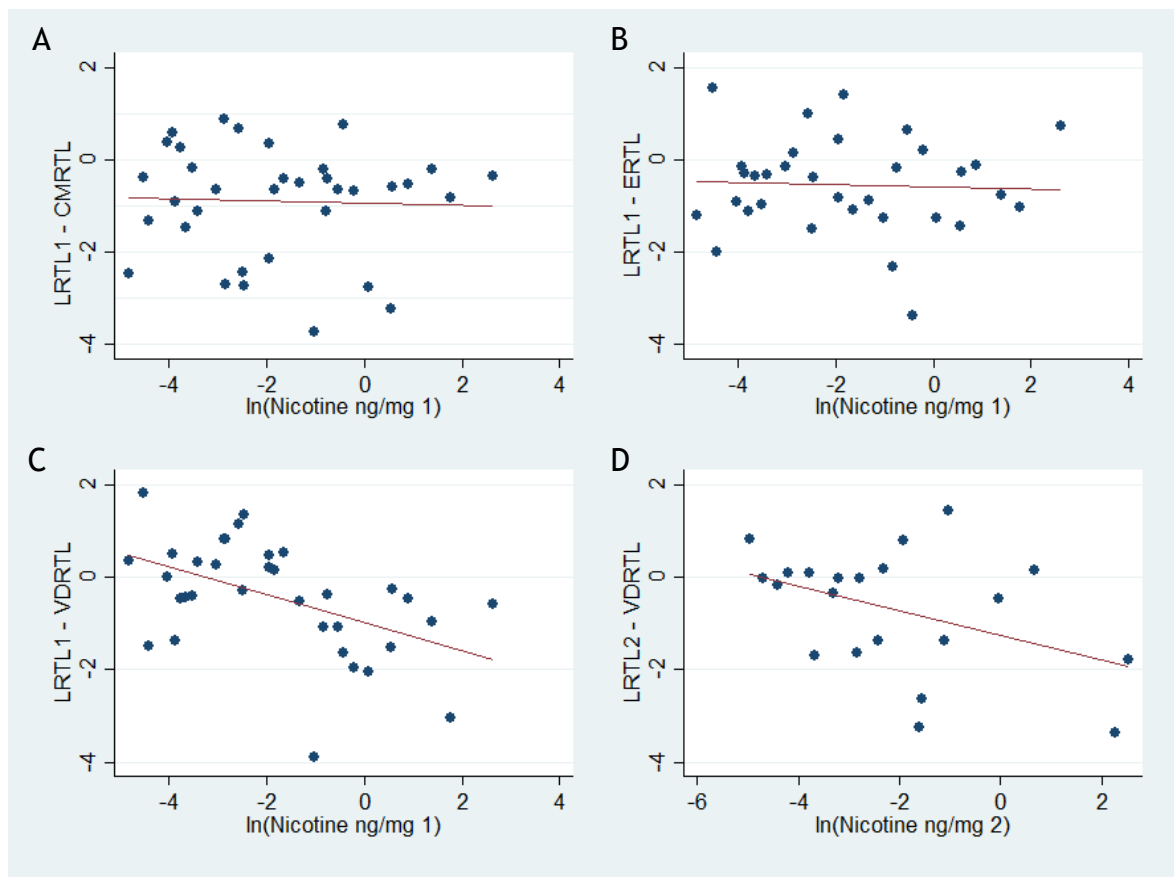


Figure 5.20 Differences in tissue telomere lengths and hair nicotine concentration

Scatterplots with regression line of difference in leukocyte RTL and cremaster muscle RTL and natural log of hair nicotine at time point 1 (A) (AIC=116.83, $p=0.82$); difference in leukocyte RTL and epididymis RTL and natural log of hair nicotine at time point 1 (B) ($R^2=0.002$, $p=0.80$); difference in leukocyte RTL and vas deferens RTL and natural log of hair nicotine at time point 1 (C) ($R^2=0.24$, $p=0.003$); and difference between leukocyte RTL at time point 2 and vas deferens RTL and natural log of hair nicotine at time point 2 (D) ($R^2=0.18$, $p=0.05$)

5.3.6 Cotinine

LRTL1, LRTL2, BRTL1 and BRTL2 were not significantly related to HCC ($p=0.33$, $p=0.08$, $p=0.42$ and $p=0.66$, respectively, figure 5.21). As with the HNC2, linear models of HCC2 and the changes in leukocyte and buccal RTL between the two time points were not significant (LRTL1-LRTL2, $p=0.20$; BRTL1-BRTL2, $p=0.35$).

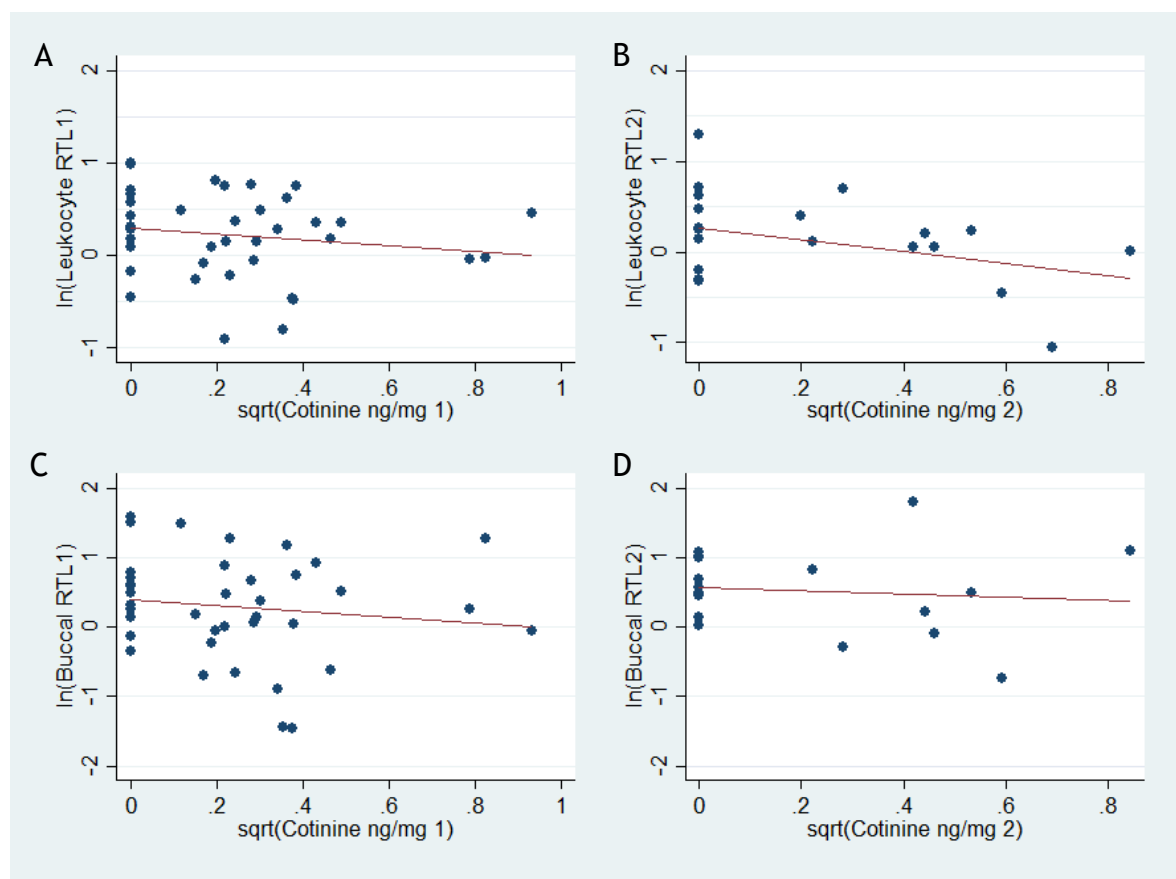


Figure 5.21 Telomere lengths by hair cotinine concentration 1

Scatterplots with regression line of natural log of leukocyte RTL and square root of hair cotinine at time point 1 (A) ($R^2=0.03$, $p=0.33$); natural log of leukocyte RTL and square root of hair cotinine at time point 2 (B) ($R^2=0.14$, $p=0.08$); natural log of buccal RTL and square root of hair cotinine at time point 1 (C) ($R^2=0.02$, $p=0.42$); and natural log of buccal RTL and square root of hair cotinine at time point 2 (D) ($R^2=0.01$, $p=0.66$)

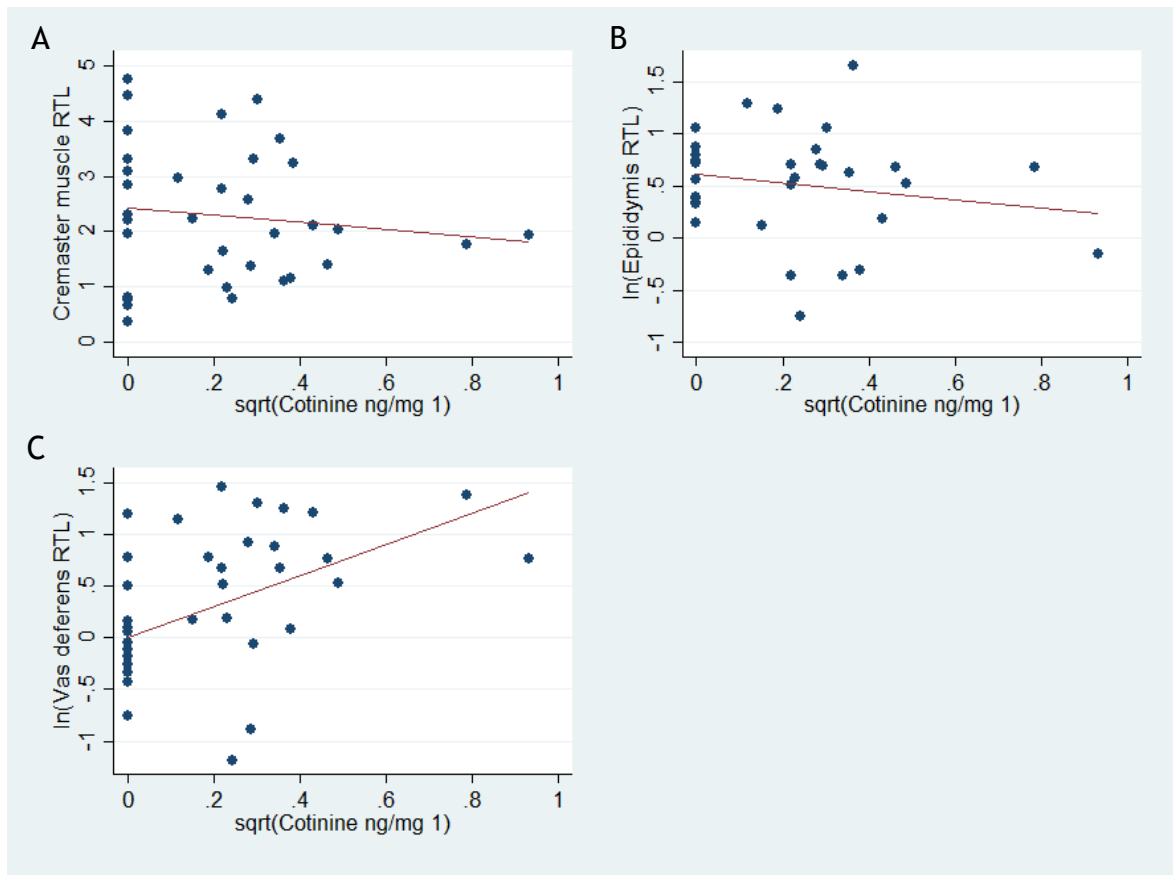


Figure 5.22 Telomere lengths by hair cotinine concentration 2

Scatterplots with regression line of cremaster muscle RTL and square root of hair cotinine at time point 1 (A) ($R^2=0.02$, $p=0.47$); natural log of epididymis RTL and square root of hair cotinine at time point 1 (B) ($R^2=0.03$, $p=0.31$); and natural log of vas deferens RTL and square root of hair cotinine at time point 1 (C) ($R^2=0.37$, $p<0.001$)

Linear models of HCC1 to CMRTL ($p=0.47$) or ERTL ($p=0.31$) were not significant. However, the linear model was statistically significant between VDRTL and HCC1 ($F=19.18_{(1,33)}$, $R^2=0.37$, $p<0.001$) (Figure 5.22). Similarly, the linear models of HCC1 to the differences between LRTL1 and CMRTL ($p=0.77$) and LRTL1 and ERTL ($p=0.98$) were not significant, but HCC1 was significantly related to LRTL1 minus VDRTL ($F=15.92_{(1,33)}$, $R^2=0.33$, $p<0.001$) (Figure 5.23). As with at time point 1, the linear models of HCC2 to the differences between LRTL2 and CMRTL ($p=0.48$) and LRTL2 and ERTL ($p=0.77$) were not significant, but there was a significant linear model between LRTL2 and VDRTL ($F=20.03_{(1,20)}$, $R^2=0.50$, $p<0.001$, Figure 5.23D).

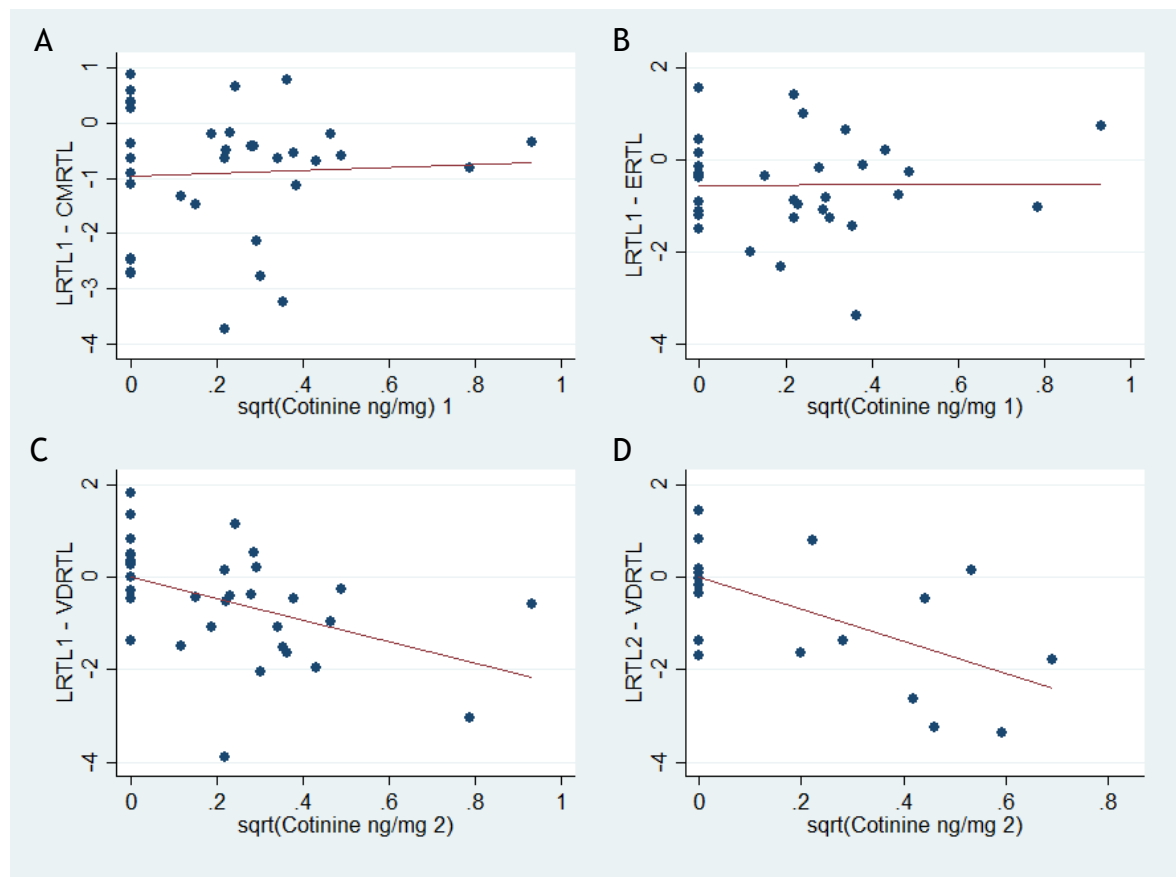


Figure 5.23 Differences in telomere lengths and hair cotinine concentration

Scatter plots with regression line of the difference in leukocyte RTL and cremaster muscle RTL and square root of hair cotinine at time point 1 (A) (AIC=116.8, $p=0.77$); difference in leukocyte RTL and epididymis RTL and square root of hair cotinine at time point 1 (B) ($R^2<0.001$, $p=0.98$); difference in leukocyte RTL and vas deferens RTL and square root of hair cotinine at time point 1 (C) ($R^2=0.33$, $p=0.0003$); difference in leukocyte RTL and vas deferens RTL and square root of hair cotinine at time point 2 (D) ($R^2=0.50$, $p=0.0002$)

HCC was not significantly modelled to the rates of change between CMRTL and LRTL1 (AIC=-30.4, $p=0.54$), ERTL and LRTL1 ($p=0.94$) or ERTL and LRTL2 per month ($p=0.06$). The rates of change between CMRTL and LRTL2 ($F=6.44_{(1,21)}$, $R^2=0.23$, $p=0.02$), VDRTL and LRTL1 (AIC=-37.24, $p=0.006$) and VDRTL and LRTL2 (AIC=-59.98, $p<0.001$) per month were significantly modelled with HCC.

5.3.7 Additional analyses

While the main focus of this chapter was on telomere dynamics with age and ETS exposure, additional analyses were carried out to investigate factors in the dogs associated with telomere length, both to inform future studies and to see if there may be interactions occurring which could impact the relationship between telomere length and ETS. These analyses are in Appendix 10.

5.4 Discussion

5.4.1 Tissue synchronicity within individuals

The first area of interest in this chapter was the degree of synchronicity in telomere length between tissues within individuals. There were few significant correlations between tissues. Interestingly, there was a positive correlation between LRTL1 and BRTL1, but no significant relationship between LRTL2 and BRTL2. This may be explained by there being fewer dogs at time point 2, or due to the factors influencing buccal telomere length which will be discussed later.

Several studies have found that telomere length in various tissues are highly correlated within individuals. TRFs of fat, muscle and leukocytes were shown to be highly correlated within individual dogs (Benetos *et al.*, 2011). Significant intra-individual correlations in telomere length in different tissues were also found in zebra finches (Reichert *et al.*, 2013), rhesus monkeys (Smith Jr *et al.*, 2011) and macaques (Gardner *et al.*, 2007). Intra-individual telomere length correlations have also been observed in newborn (Okuda *et al.*, 2002; Kimura, Gazitt, *et al.*, 2010) and adult humans (Granick *et al.*, 2011; Daniali *et al.*, 2013). One study was found with few correlations between telomere length in tissues obtained from human cadavers (Dlouha *et al.*, 2014), but this study was certainly in the minority in terms of intra-individual observations. It is unclear why there were few correlations between tissues found in this study. This may have been due to some of the dogs growing and undergoing rapid telomere length changes. By adding in an interaction with biochemical or haematological measures to account for some of the physiological changes which may have been occurring, there were improved relationships seen between the tissues. For example, a linear model of LRTL1 and albumin:globulin ratio to CMRTL was highly significant ($F=54.38_{(3,32)}$, $R^2=0.84$, $p<0.001$). When LRTL2 and the change in monocyte count between the two time points was regressed to LRTL1, the relationship was significant ($F=5.44_{(2,21)}$, $R^2=0.34$, $p=0.01$). Growth may have also accounted for, in part, the lack of consistency in individuals rank position of LRTL between the two time points. Benetos *et al.* (2013) found that in 1156 adult individuals with multiple LTL

measurements, 94.1% kept their decile rank or only changed one place. Unlike here, only fully developed adults were included in their study. Their observations were also on a much higher number of individuals.

5.4.2 Longitudinal telomere length measurements

Neither LRTL1 and LRTL2 or BRTL1 and BRTL2 were significantly correlated, with individuals not retaining their rank position within the group for either tissue, i.e. those with the longest LRTL1 or BRTL1 did not necessarily have the longest LRTL2 or BRTL2. 8 (32%) individuals had longer LRTL2 than LRTL1. Telomere lengthening would not have been expected based on telomere physiology. However, this study is not alone in having this occur in longitudinal telomere length studies. The rate of lengthening was similar to that in Nordfjäll *et al.* 2009, who found that in measures 10 years apart, one third of LTLs were either stable or increased. Steenstrup *et al.* (2013) suggested that leukocyte telomere lengthening is an artefact and due to factors such as short follow up periods or not using high precision methods of measurement. It was found that there was a difference in haematological measures between shorteners and lengtheners which may have reflected some underlying physiology. It was difficult to schedule time points exactly one year apart for logistical reasons. 5 dogs attended their time point 2 appointments a month early; 4 of these dogs were in the lengthener group. While this is not a huge difference from a year, Rehkopf *et al.* (2014) noted seasonal variation in LTL in humans. There was not enough of a spread over the year to estimate if having samples taken in different months would have had such an impact. Only one study could be found which examined the link between clinical pathology results and telomere length elongators (Kato *et al.*, 2016). They found that in 59 dialysis patients, telomere length elongators had a higher baseline white cell count. This finding was not replicated here, but change in monocyte count was. Interestingly, there were a few health problems reported in some of the elongators. One of the dogs had received steroid treatment. Another dog had received behavioural treatment for an aggression problem. Given the differences in haematology measures between the two groups, the slide reports were reviewed. Polychromasia was present in 3 of the dogs in the L group. The

combination of the L group having higher MCV and MCHC, as well as polychromasia on some of the dogs' blood films, implies that these dogs may have had a greater proportion of reticulocytes, although not at pathological levels. It could be related to their diet, for example vitamin B12 intake. Without nutrient analysis of the blood, this cannot be confirmed. Telomerase levels have been linked to diet in previous studies. Higher fat intake has been linked to higher telomerase activity in human leukocytes (Daubenmier *et al.*, 2012). Therefore, a contributing factor could be diet. It may have been worth asking about the diet the dogs were fed in the questionnaire, although there would have been a wide variety of brands, quantities fed and supplementation with treats. In addition, the dogs were likely fed a commercial diet, which is more standard than a human diet. It is only possible to postulate reasons, with the small numbers involved and lack of longitudinal canine telomere studies published.

The change in LRTL depended on baseline LRTL, with individuals with longer LRTL1 losing more bases than those with shorter LRTL1, as found in other studies, for example Farzaneh-Far *et al.* 2010 and Weischer *et al.* 2014. This remained the case after correction for regression to the mean, which Verhulst *et al.* 2013 also found. Therefore, the dependence of the rate of change on the baseline was only in part due to a statistical phenomenon. This was also the case for buccal cell telomere lengths. In addition, as part of this relationship, the individuals with the shortest telomere lengths at the first time point tended to experience lengthening between the two time points.

5.4.3 Telomere length relation to age

The second key area of this chapter was to assess which tissue or combination of tissues would best represent age. Contrary to expectations, the buccal and leukocyte telomere length measures did not reduce with age, and in fact the buccal telomere measures at time point 2 increased with age. In addition, after correcting for breed life expectancy, there was a trend for leukocyte RTL1 to increase with age. However, the negative relationship expected was found

between buccal RTL1 and life expectancy adjusted age. Part of the reason for the lack of age-related changes expected may be partly due to the wide range of breeds and crosses, with a corresponding range of life expectancies. With the presence of crossbreeds and less common breeds in the study, such as the Patterdale terrier, not all dogs age could be corrected for life expectancy, further limiting the number available for statistical analysis. Issues with life expectancy as a measure in dogs were discussed in Chapter 4 discussion. As well as there only being a small number of participants, there was a skew to the younger ages. Multiple studies have found that the rate of leukocyte telomere loss is greatest at the younger ages of humans, and in one study dogs (Frenck Jr, Blackburn and Shannon, 1998; Zeichner *et al.*, 1999; Benetos *et al.*, 2011). In addition, different breeds have different rates of growth (Hawthorne *et al.*, 2004). Therefore, with many the dogs being younger and some still growing, this may be why a linear model of age related telomere attrition in leukocytes was not able to be captured. In addition, a wide inter-individual variation in LTL has been displayed in several previous studies (e.g. Slagboom, Droog and Boomsma, 1994; Factor-Litvak *et al.*, 2016) and the variation between individuals may have been too great to capture underlying age related changes.

As a means of attempting to overcome the issue of inter-individual variability in LTL, telomere length was also measured in a post mitotic tissue, the cremaster muscle. The cremaster muscle telomere length was meant to represent the telomere length at birth, and the difference between the two the loss since birth (assuming all tissue telomere lengths correlated well within individuals at birth). Vas deferens was also trialled as a comparative tissue, as while smooth muscle is capable of undergoing mitosis if damaged, the use of skeletal muscle as an internal control has been critiqued and only one study had previously used cremaster muscle for this purpose in dogs (Benetos *et al.*, 2011). Therefore, to test another tissue from those available from castration seemed prudent. For this model of telomere length dynamics to work as hypothesised, the telomere length in the muscle should not change with time. The linear models of cremaster muscle telomere length to age and life expectancy adjusted age were not significant.

However, the linear model of life expectancy adjusted age to vas deferens telomere length was highly significant.

The pattern of telomere dynamics with age in the epididymis was similar to that of the vas deferens, with a significant decrease with life expectancy adjusted age. It was hoped to use the epididymis samples as a proxy for spermatozoa samples due to practical issues faced collecting spermatozoa alongside the other samples at the point of castration. Fradiani *et al.* (2004) demonstrated that, in pigs, spermatozoa isolated from the corpus, cauda I, cauda II and ejaculated spermatozoa TRFs were similar in size, with testis and caput TRFs slightly longer. Their sample preparation method differed from that here: they released sperm cells from small pieces of the tissues with cold PBS and used the solution. The method used here resulted in the inclusion of epididymal tissue along with any spermatozoa present and did not give the result anticipated (that telomere length would increase with age due to the nature of telomere length dynamics in sperm), so adopting a method such as that used by Fradiani *et al* may be beneficial in future studies.

Cremaster muscle telomere lengths were significantly longer than leukocyte telomere lengths at both time points and buccal telomere lengths at time point 1, and non-significantly longer than the remaining tissues. Skeletal muscle having the longest telomere length was expected and has been shown in other studies. Epididymal telomere lengths were longer on average than leukocyte telomere lengths at both time points, and vas deferens telomere lengths were non-significantly longer than leukocyte telomere lengths. High turnover tissues, like leukocytes and skin, have been shown to have the shortest telomere lengths (Smith Jr *et al.*, 2011; Daniali *et al.*, 2013). What was unusual here is with increasing life expectancy adjusted age, the difference between telomere length in vas deferens and epididymis and leukocyte telomere length was approaching zero. In addition, this reduction in difference with increasing adjusted age was also seen between cremaster muscle and leukocyte telomere lengths.

No studies could be found examining age related changes of vas deferens telomere length, or epididymis telomere length (except the spermatozoa telomere length contained within). The largest component of both epididymis and vas deferens is layers of smooth muscle. Many smooth muscle telomere length studies examine smooth muscle in the context of vascular tissue in the context of atherosclerosis, which would not be relevant here. Various organ telomere lengths have been measured in the context of age-related change. For example, changes in thyroid and lung, but not kidney cortex, kidney medulla or testis with age were demonstrated in macaques (Gardner *et al.*, 2007); reduction with age was shown in liver, renal cortex and spleen, but not cerebral cortex or myocardium in human cadavers (Takubo *et al.*, 2002); and percentage of short telomeres was shown to increase with age in kidney, liver, pancreas and lung of male and female rats (Cherif *et al.*, 2003). Gastrointestinal telomere length may behave similarly to that of vas deferens and epididymis, having also a tube-like structure for the most part and the presence of smooth muscle. A significant reduction in telomere length with age was found in duodenum samples, but not stomach, blood or colon in a group of human patients undergoing upper or lower GI endoscopy (Craig, McKinlay and Vickers, 2003); which is particularly interesting as duodenum would be the most comparable to vas deferens out of the four and the relationship with age was stronger in duodenal telomere length than blood telomere length. Another factor which could be involved in epididymis and vas deferens telomere length changes is puberty and hormonal changes. Interestingly, when 42 pairs of epididymes from healthy cats aged 6 months to “7+” years undergoing castration were examined, 70% of cats 6 months to 1 year-old displayed epididymal intraepithelial cysts and epithelial hyperplasia. Other age related changes were uncommon, bar increasing thickness of tunica adventitia with age (Elcock and Schoning, 1984). Peri-pubertal changes in dogs in the current study could have occurred and potentially cause telomere shortening in the epididymis and vas deferens.

Cremaster muscle telomere length was found not to significantly change with dogs in a previous study (Benetos *et al.*, 2011), which was repeated here. Telomere

length in the various tissues did not shorten at equivalent rate to different tissues as found in Daniali et al. (2013), who found that skeletal muscle shortened at a similar rate as leukocytes, skin and fat. Part of the criticism of the thinking that telomere length in skeletal muscle does not change with age is that *in vivo* it is part of a dynamic system. Satellite cells are the proliferative cell type within skeletal muscle, and can be activated and proliferate due to a variety of factors, including hormone changes and environmental factors. Satellite cell activation can be associated with changes in telomere length (Kadi and Ponsot, 2010). The fact that many of the dogs were peri-pubertal therefore is very interesting given the potential hormonal impact on satellite cells. There are published examples of satellite cell induction through testosterone treatment. Satellite cells in rat levator ani muscle were induced by testosterone injection (Joubert and Tobin, 1995). In addition, testosterone treatment in elderly men induced satellite cell proliferation (Sinha-Hikim *et al.*, 2006). In addition, skeletal muscle satellite cells have also been shown to be reduced in cigarette smoke exposed mice (Jackson *et al.*, 2013). Satellite cell activation is part of the notch pathway, which has been shown to be impacted by smoking (e.g. Tilley *et al.* 2009). So, while there was not a significant relationship between cremaster muscle telomere length and age, cremaster muscle telomeres are certainly under the influence of environmental and hormonal changes.

While the findings regarding single tissue telomere length changes and age were not encouraging to use telomere length as a biomarker of age, it was still tested to see whether the combination of two tissues better represented age, and if findings from previous studies could be replicated. The differences between leukocyte and vas deferens telomere lengths were not significantly modelled to age and the difference between leukocyte and epididymis telomere lengths was only modelled to age at the second time point. As previously mentioned, however, for all the life expectancy adjusted age to leukocyte minus cremaster muscle, epididymis or vas deferens telomere lengths, the regression line approached zero, indicating a reduction in difference over time.

There was no perfect age reflecting tissue found. The change between leukocyte and cremaster muscle telomere length seemed to reflect age best out of the tissue combinations. Adjusting age for life expectancy improved fit of models but limited the dogs which could be included for analysis, given the limited information on data on lifespan for some breeds and cross-breeds, and would limit the potential for dogs being recruited in future studies if that form of analysis was opted for.

5.4.4 Telomere length association with ETS exposure

Having not found a change in age with leukocyte telomere length at time points 1 or 2, there was a significant decline with HNC at time point 1 and a near significant decline at time point 2. Buccal telomere length at time points 1 and 2 also shortened with increasing HNC. Correcting for HNC did not improve relationships with age. Contrary to previous studies examining the impact of smoking on human telomeres, ETS exposure, as determined by HNC, had a larger effect on leukocyte telomere length than age did in these dogs. HCC was not significantly related to the leukocyte or buccal telomere lengths, unlike with HNC. Part of the issue was that, unlike with HNC, there were several zero HCC values, which limited the linear modelling analysis. If higher numbers of participants were involved, HCC may have been significantly related to the leukocyte and buccal telomere lengths.

Interestingly, vas deferens telomere length increased with ETS exposure. There have been studies previously where telomere length has increased due to smoking (Appendix 7), but the increase here occurred on a background of other tissues within the same dogs decreasing with ETS exposure. A reason may be that telomerase is being activated in this tissue as a result of the exposure. No studies could be found regarding telomerase levels in the vas deferens, ETS exposure or not. However, telomerase reverse transcriptase expression was found to decrease in the skin of smokers (Lotfi *et al.*, 2014) and the lymphocytes of smokers with Crohn's disease (Getliffe *et al.*, 2005). It may be that the effect is tissue

dependent, the information is too limited to know what is happening throughout the body. This lengthening of telomeres may initially appear attractive, with reduced cellular senescence and the opposite of the proposal that smoking shortens telomere length. However, this increase of cell proliferative potential is a potential cancer risk. Telomerase activity has previously been detected in canine mammary tumour samples (Yazawa *et al.*, 2001). It would be interesting to see if telomerase activity levels in different tissues throughout the body are affected by ETS exposure, and if malignancy development is affected by this.

The differences in leukocyte and vas deferens telomere lengths were significantly related to markers of ETS exposure, however this was likely due to the large effect on vas deferens telomere length rather than the tissue combination being a useful model. The other tissue comparisons were either not related to ETS exposure, or required the inclusion of further explanatory variables to be significant. While inclusion of a second tissue telomere length, namely cremaster muscle telomere length with leukocyte telomere length may have helped establish an age marker, leukocyte and buccal telomere lengths relationships with HNC alone adequately displayed the effects of ETS exposure.

Buccal telomere length decreased with increasing age at time point 1, yet increased with age at time point 2. Previous findings in chapter 4 indicated that entire males had significantly different oral telomere length. The change in buccal telomere length in this chapter also points to the same finding, with more dogs experiencing telomere lengthening than shortening between the two time points. It appears there may be an effect of testosterone on buccal cell telomere length. Perhaps the increase with age after castration is that the remaining testosterone, produced by the adrenal gland, decreases with age. In addition, as shown in Chapter 7, the dogs with less testosterone at time point 2 gained more weight. This may explain the increasing buccal telomere length with additional weight gain model.

A highly interesting finding on further analysis was the positive link between weight and cremaster muscle telomere length. In addition, weight was positively associated with leukocyte telomere length at time point 1, buccal telomere length at time point 1, epididymis telomere length and vas deferens telomere length. The differences between leukocyte and cremaster muscle or epididymis telomere length were negatively associated with increasing weight. It appears from the information gathered that larger dogs start life with longer telomeres and have a faster rate of telomere loss. Obviously, some dogs were not their ideal weight, and where dogs gained weight above that expected as part of normal growth between the two time points, the difference between leukocyte and cremaster muscle telomere lengths increased. Height was also positively linked to telomere length, but measures of height for breeds were obtained from other sources. An idea for future studies would be to take measurements of height and leg length for each dog. However, with imperfect explanatory variables, a strong relationship with cremaster muscle telomere length both weight and height was demonstrated. A possible reason for longer CMRTL in larger dogs is a positive relationship between telomere length and IGF-1 levels, which has been demonstrated in previous studies (e.g. Barbieri *et al.* 2009; Movérare-Skrtic *et al.* 2009), with IGF-1 being a key determinant of dog size (e.g. Greer *et al.* 2011; Sutter *et al.* 2007). If the general, although imperfect, rule of large dogs have shorter life expectancies is applied here, the greater rate of loss in larger dogs would be in keeping with other studies which have found that telomeres shorten more slowly in long-lived mammals (reviewed in Haussmann *et al.* (2003)). Previous studies in dog telomeres have not commented on the relationship with weight. The idea of larger dogs having longer telomeres initially goes against the idea of smaller animals having longer telomeres (Gomes *et al.*, 2011), however mostly this rule is talked about at a species level rather than at a breed level. At time point 2, leukocyte telomere length could be significantly modelled to breed life expectancy and vice versa (generalised model of LRTL2 to life expectancy, $p=0.02$). At time point 1, life expectancy can be significantly modelled to leukocyte telomere length, but leukocyte telomere length cannot be used to significantly explain life expectancy. The findings at time point 2 are in keeping with those of Fick *et al.* (2012), who found a highly significant linear regression of telomere length to life span.

Multiple statistical tests have been performed in this chapter without p value correction. This could have led to many Type-1 errors. However, given that this study is to be used for pilot data to inform future studies, it seemed prudent fully explore the implication of physiological changes with growth and changes in the body due to smoking, and how this was associated to linear models of telomere length. By correcting the p value, this increases the Type 2 errors and interesting biological relationships could be overlooked. The strong relationship between cremaster telomere length and dog weight, for example was not expected.

5.4.5 Chapter summary

This chapter aimed to find the best tissue combination of telomere lengths to provide a biomarker of ageing, to assess change in biological age caused by ETS. While the best marker of ageing was provided by using the difference between cremaster muscle and leukocyte telomere length, this was then not actually the best means of evaluating ETS exposure impact.

Nicotine exposure impacted leukocyte and buccal telomere length at both time points (time point 2 leukocyte close to significance). Age related changes were not observed in leukocyte telomere lengths, yet a clear relationship with nicotine could be seen in the plots. Little extra information was obtained by having the change between these measures, and like most studies, fewer individuals attended at the second time point. A review of previous work did not find that longitudinal telomere length studies were any more likely to find an impact of ETS, and therefore future studies could be simplified using a single time point. The use of nicotine as a biomarker of ETS exposure was a useful measure, as it provided an objective way of measuring exposure that enabled linear modelling analysis. Without having a larger study population, it is not clear that having cotinine as a second measure added anything to the study.

Using a method similar to that used by Benetos *et al* (see Chapter 1) did not improve illustration of age-related change in telomere length until the second time point, when numbers were smaller. The cohort may have been too young at time point 1 for the model to be utilised, or the TRF analysis method of measurement may have been required to generate the necessary data. It was not expected for there to be such a strong relationship between cremaster telomere length and weight, which also may have affected the usefulness of this measure.

Overall, it has been demonstrated in this chapter that there appears to be a relationship between ETS exposure and telomere length. Further work on a larger scale using this information would be worthwhile, to both provide owners with evidence as to the potential impact their health behaviours may have on their pet's welfare, and to inform potential human studies based on the parallels between canine and human telomere dynamics. However, given the overall aim of this thesis was to demonstrate whether an increase in biological age occurs as a result of ETS exposure, further potential biomarkers were investigated and are described in Chapter 6.

Chapter 6: Testicular gene expression and leukocyte DNA methylation as biomarkers of ageing

6.1 Introduction

Aside from telomere length, several other potential ageing biomarkers have been investigated. This chapter examined markers which have either been examined in previous studies or could be biologically plausibly expected to change with age. Summarised below is the previous evidence regarding tobacco smoke exposure in the markers which were investigated.

6.1.1 CDKN2A/p16^{INK4a}

CDKN2A is a marker of cell senescence and mRNA expression levels have been shown to increase with age (e.g. Krishnamurthy *et al.* 2004). CDKN2A was selected to study here as, along with telomere length, it is one of the two proposed biomarkers of ageing which have met the majority of the Baker III & Sprott (1988) criteria for a biomarker of ageing. As well as increasing with age, CDKN2A mRNA levels were shown to increase with pack years of smoking in peripheral T lymphocytes of 128 human volunteers (Liu *et al.*, 2009). Nyunoya *et al.* (2006) demonstrated that cigarette smoke upregulated the p16-retinoblastoma protein pathway in a human lung fibroblast cell line. They also observed that when the cells were exposed to cigarette smoke concentrate over a time course, cell growth was inhibited at multiple cell cycle phases, but the cells did not die. However, in a study examining bronchioles and parenchyma of C57BL/6 female mice exposed to cigarette smoke for 6 months, there was no difference observed in CDKN2A expression due to exposure compared to a control group exposed to air in young or old animals (Zhou *et al.*, 2013).

6.1.2 Sirtuin 1

Sirtuins are a group of proteins, several of which have been shown to change with age. Of these proteins, sirtuin 1 was chosen. This was decided because sirtuin 1 has previously been measured in dogs and cats (Ishikawa *et al.* 2015; Marfe *et al.* 2012), it has been shown to be expressed in all tissues (including testes)

(Michishita *et al.*, 2005), and it is the most widely studied in mammals. In addition, an interaction has been observed between sirtuin 1 and telomerase (Narala *et al.*, 2008) and it would be interesting to explore whether a relationship was seen in the dogs in this study. Sirtuin 1 has been shown to both reduce with age (e.g Donato *et al.* 2011) and as a result of cigarette smoke exposure. Lung tissue from 37 subjects undergoing lung surgery demonstrated decreased sirtuin 1 protein concentrations in lungs of smokers and COPD patients compared to non-smokers (Rajendrasozhan *et al.*, 2008). In the same study, a decrease in sirtuin 1 concentration was seen in macrophages, alveolar epithelial cells and airway epithelial cells in smokers and COPD patients compared to non-smokers. In addition, Yang *et al.* (2007) demonstrated a decrease in sirtuin 1 due to cigarette smoke exposure in monocyte-macrophage cells (Mono Mac 6 cells) and bronchoalveolar cells of Sprague-Dawley rats exposed in chambers to cigarette smoke. In contrast, Csiszar *et al.* (2008) measured sirtuin 1 in twenty 14-16 week old Wistar rats exposed to cigarette smoke for one week and found no difference to controls, unless smoke exposed rats were treated with resveratrol, an inducer of sirtuin 1.

6.1.3 AHR

AHR is activated by xenobiotic ligands, which include chemicals found in cigarettes, such as polycyclic aromatic hydrocarbons (PAHs). The downstream pathway components not only include p450 enzymes for metabolism of the ligand, but also genes involved in the cell cycle. This gene was selected for this study because not only is its activity directly affected by smoking, but it is also involved in processes related to biological ageing. In addition, the metabolism of PAHs by p450 enzymes has also been shown to sometimes result in the production of reactive oxygen species (Puntarulo and Cederbaum, 1998), and therefore negative downstream effects. Hepatic AHR in beagles was shown to have similar binding for [3H]-2,3,7,8-tetrachlorodibenzo-p-dioxin to that shown by cynomolgus monkeys, and the binding seemed to be comparable to that of humans for both species. Therefore, the dog could work well as a model organism for humans for AHR

biology when exposed to ETS in the home. In addition, AHR is expressed in germ cells (Coutts, Fulton and Anderson, 2007) so can be measured in testicular tissue.

When mouse spermatocyte cell line GC-2spd cell line was cultured, cigarette smoke concentrate (CSC) produced a dose dependent increase in AHR mRNA expression. CSC mediated changes in other genes and proteins were examined. When an antagonist to the AHR was included, several of the CSC-mediated changes were rescinded (Esakky *et al.*, 2012). In addition, when blood samples from 20 healthy adult humans were examined, the concentrations of AHR and AHR nuclear translocator (Arnt) increased with smoking in a dose-dependent manner (Hayashi *et al.*, 1994). They combined AHR and Arnt in this analysis, so it is not clear whether individually AHR was related to smoking intensity, however, it was shown in this study that AHR and Arnt expression was significantly positively correlated. In contrast, a reduction in AHR mRNA level was observed in human uterine endometrium samples taken from smokers, compared to non-smokers in a group of 30 women undergoing surgery for benign disorders (Igarashi *et al.*, 1999). Fowler *et al.* (2014) examined the effect of maternal cigarette smoking on mRNA expression levels in human female foetal ovaries. Foetuses were collected from elective termination of pregnancy and were of 11 to 21 weeks' gestation. In 22 foetuses studied, there was no significant difference in the level of AHR expression with maternal smoking status. However, they also measured the expression of AHR associated and response genes and found that for COUP (chicken ovalbumin upstream promoter) transcription factor 1, a gene with multiple AHR binding sites, mRNA levels were significantly higher in smoke exposed foetuses. As with these foetal ovaries, when 22 human male foetal testes were examined, there was no significant difference in AHR mRNA levels with maternal smoking (Fowler *et al.*, 2008). Similarly, in 14 full term human placentae, there was no significant difference in AHR mRNA expression level between smoking and non-smoking mothers (Huuskonen *et al.*, 2016). Also, Lin *et al.* (2003) measured AHR expression in cultured lymphocytes collected from healthy adult humans, and found no difference in expression level based on smoking status.

6.1.4 Clusterin

Clusterin is increased in the presence of stress-inducing agents and senescence, and helps prevent apoptosis. Mixed evidence exists as to its changes with ageing, but study numbers are limited. This gene was chosen because it has been previously measured in dogs (as a renal marker) (García-Martínez *et al.*, 2012), it is present in testes (Fritz *et al.*, 1983) and while its mechanisms are said not to be fully understood, it has been studied in the context of diseases for which smoking and/or ETS exposure are risk factors, for example, coronary heart disease (Poulakou *et al.*, 2008) and childhood asthma (Sol *et al.*, 2016).

Carnevali *et al.* (2006) exposed human lung fibroblasts to cigarette smoke extract (CSE). Clusterin mRNA and protein levels were significantly upregulated after exposure to 2.5% and 5.0% CSE after 72 hours. Clusterin appeared to protect against cigarette smoke-induced oxidative stress in these cells. In another study, peripheral blood mononuclear cells were isolated from blood samples collected from 16 healthy adult human male donors and the cells were differentiated into monocyte-derived macrophages. Clusterin mRNA expression was measured in these monocyte-derived macrophages, and expression level was found to be 2.1 times higher in smokers than non-smokers (Doyle *et al.*, 2010). Furthermore, when Yanni *et al.* (2014) obtained carotid artery samples from 42 human patients undergoing surgery for carotid artery stenosis, density of clusterin expression on carotid tissue was significantly higher in smokers than non-smokers. When serum clusterin protein concentration was measured in 183 human patients undergoing coronary angiography, there was a non-significant trend for the clusterin levels to be higher in smokers. Additionally, when plasma clusterin protein concentration was measured in 204 human adults attending a health screen, there was no significant difference in percentage of smokers between quartiles of clusterin concentration, however the highest percentage of smokers were in the highest quartile of clusterin concentration (Won *et al.*, 2014). In contrast, when relative clusterin content in red blood cell membrane was measured in blood samples from 45 healthy adult humans, smokers had significantly lower clusterin levels (Antonelou *et al.*, 2011).

6.1.5 TERT

TERT, an element of the telomerase enzyme, was selected for this study as telomerase activity has previously been measured in dog testis (Nasir *et al.*, 2001), human and canine TERT promoters behave in an equivalent manner (Arendt, Nasir and Morgan, 2010) and it was interesting to examine the relationship between TERT and telomere length. Due to the small number of studies found, examples of the relationship between smoking and both TERT and telomerase level and activity will be presented here.

When telomerase activity was determined in bronchial epithelial cells obtained from 26 humans, telomerase activity increased with increasing pack years smoked (Yim *et al.*, 2007). In addition, when leukocyte telomerase activity was measured in 440 adult men, smoking was associated with higher telomerase activity (Kroenke *et al.*, 2012). Whereas, when TERT relative expression was measured in blood samples from 334 male lead smelters, there was no association between smoking and TERT expression (Pawlas *et al.*, 2016). In addition, when telomerase activity was measured in leukocytes from 217 humans of mean age 77.9, there was no relationship between smoking habit and telomerase activity when analysed as part of a multivariate model. In contrast, when TERT was measured in skin punch biopsies from 20 current smokers and 20 non-smokers, pack years of smoking was inversely correlated to TERT expression in the epidermis (Lotfi *et al.*, 2014). Getliffe *et al.* (2005) measured TERT mRNA expression levels in activated lymphocyte samples from 15 human adults with ulcerative colitis, 14 adults with Crohn's disease and 15 controls. In those with Crohn's disease, smoking was associated with significantly lower TERT expression. In the other two groups, smoking non-significantly lowered TERT expression.

6.1.6 Steroidogenic pathway

mRNA levels of 3BHSD, 17BHSD, p450 SCC and StAR were measured. As well as age related change being demonstrated elsewhere (e.g. Luo, Chen and Zirkin, 1996; Leers-Sucheta, Stocco and Azhar, 1999), it was decided to measure these genes as the tissue being used was testes, so high levels of these genes were expected to be present. In addition, the ability to measure these genes in testes would be an interesting model for human fertility, as it would be problematic (and no doubt objectionable to men) to obtain testis tissue from humans in a home environment to investigated relationships with ETS exposure.

Jana *et al.* (2010) subjected 4 month-old inbred male Wistar rats to 12 weeks of daily nicotine injections. The rats were then euthanised and testicles harvested. Nicotine treatment resulted in a significant decrease in 3BHSD and 17BHSD activity compared to control rats. In addition, there was a significant decrease in StAR expression in nicotine exposed rats. In another study, female mice were exposed to cigarette smoke from 8-9 weeks of age for 4 hours a day, 5 days a week, for 14 weeks. The mice were then euthanised and the adrenal tissues collected. Mitochondria were isolated from the adrenal tissues and StAR import measured. StAR is required for the movement of cholesterol to the inner mitochondrial membrane for steroidogenesis. Mice exposed to cigarette smoke demonstrated a reduction of StAR import compared to control unexposed mice. As a result, cigarette smoke showed a reduction in pregnenolone synthesis (Bose *et al.*, 2008). Yan *et al.* (2014) subcutaneously administered nicotine to pregnant Wistar rats from gestational days 11 to 20. The feto-placental units were removed and RNA isolated from foetal adrenal glands. The expression of StAR and 3BHSD mRNA were inhibited by maternal nicotine administration compared to controls. p450 SCC mRNA expression was also inhibited, but not significantly. In further experiments, they also treated human adrenal carcinoma cells with nicotine. Total RNA was isolated. StAR and p450 SCC mRNA were reduced with increasing concentrations of nicotine, but there was no difference in 3BHSD mRNA with nicotine concentration.

In 3 further studies, human foetal mRNA expression levels in relation to maternal smoking were examined. Foetal material was collected in all studies from elective termination of pregnancies, between 11 and 21 weeks' gestation. When RNA was extracted from 22 foetal ovaries, p450 SCC was significantly reduced as a result of maternal smoking, whereas StAR, 17BHS type 3 (the major type in males) and 3BHS type 2 (the type found in testes) were non-significantly reduced with maternal smoking (Fowler *et al.*, 2014). In RNA from 22 foetal testes, there were non-significant decreases in StAR and p450 SCC expression levels with maternal smoking, and little change in 17BHS type 3 expression (Fowler *et al.*, 2008). In mRNA from 55 foetal livers, there was no significant change in p450 SCC or 17BHS type 3 with maternal smoking status (O'Shaughnessy *et al.*, 2013). Huuskonen *et al.* (2016) examined RNA expression levels in 14 full-term human placentae, and found that there was no significant difference in 3BHS type 2 or p450 SCC expression level with maternal smoking.

Changes in testosterone concentration itself relating to ETS exposure will be discussed in Chapter 7.

6.1.7 Global DNA methylation

Global DNA methylation, the percentage of cytosine residues modified to 5-methylcytosine, has been shown to decrease with age (e.g. Wilson and Jones, 1983). It was measured in this study as it can be examined longitudinally in a variety of tissues. LINE-1 constitutes approximately 17% of the human genome (Cordaux and Batzer, 2009), so methylation of LINE-1 is regarded as a surrogate measure of global DNA methylation. Therefore, examples of studies examining the relationship between smoking and both global DNA methylation and LINE-1 methylation will be discussed here.

Ivorra *et al.* (2015) measured global DNA methylation in cord blood samples from 20 new born human babies. They found that infants exposed to maternal smoking

during pregnancy had significantly reduced global DNA methylation. Similarly, when Guerrero-Preston *et al.* (2010) measured global DNA methylation in 30 cord blood samples, there was a reduction in global DNA methylation with maternal smoking, but there was also a reduction in global DNA methylation when the mother was exposed to passive smoking. In addition, cord blood cotinine concentration was inversely related to global DNA methylation. Whereas, when LINE-1 methylation in buccal scrapes from children was measured, there was no difference in level based on prenatal tobacco smoke exposure, but there was a significant difference in AluYb8 methylation based on prenatal tobacco smoke exposure (Breton *et al.*, 2009). Like LINE-1, AluYb8 is also a repetitive element in the human genome (Cordaux and Batzer, 2009).

In DNA from non-cancerous oesophageal mucosa samples from 109 patients, a reduction in LINE-1 methylation was found with both years of smoking and increased number of cigarettes per day (Shigaki *et al.*, 2012). Whereas, in leukocyte DNA from newly diagnosed breast cancer patients plus controls, there was no significant difference in global DNA methylation with smoking status or number of pack years (Choi *et al.*, 2009). In addition, when global DNA methylation and hydroxymethylation were measured in blood samples from 48 healthy adults of mean age 54.9, there was no significant difference with smoking status in either measure (Tellez-Plaza *et al.*, 2014). When global DNA methylation was measured in 298 DNA samples from a group of parents and offspring, there was no significant difference in global DNA methylation between smokers and non-smokers. In addition, within smokers there was no significant relationship between pack years of smoking and global DNA methylation (Hillemacher *et al.*, 2008). Flom *et al.* (2011) measured LINE-1 methylation in 90 blood samples from the New York Women's Birth cohort. They found no significant difference in LINE-1 methylation between those exposed prenatally to cigarette smoke or not, or in adult smoking status.

6.1.8 Chapter aims and hypotheses

Multiple gene expressions were measured in the testes of study dogs to establish if any changed with age and/or ETS exposure. In addition, global DNA methylation was measured in leukocyte DNA to see if this could be a viable biomarker of ageing in dogs.

Firstly, protocols for measurement of relative gene expressions were established and mRNA expression levels of genes measured in cDNA samples from testes. Global DNA methylation in leukocyte DNA samples was measured using an ELISA kit. Gene expression of steroidogenic enzymes: 3 β HSD, 17 β HSD, StAR and p450 SCC were expected to decrease with both age and ETS exposure as part of leydig cell ageing. CDKN2A and clusterin expression were hypothesised to increase with age and ETS exposure, due to their link with senescence. Whereas, sirtuin 1 and global DNA methylation were hypothesised to decrease with age and ETS exposure. Both AHR and TERT mRNA levels were expected to decrease with age, but to increase with ETS exposure as an attempt to protect from the damage being caused.

6.2 Methods

6.2.1 Samples

Details regarding owner and dog demographics and ETS exposure were as in chapters 3 and 5. Leukocyte DNA used was from the same DNA extracted for assays in chapter 5. Testes were obtained from the dogs detailed in chapter 5. In addition, testes were obtained from a 1 year old and a 3 year old dog (both cross breeds) courtesy of the SSPCA, which were used for a comparative sample and for inter-assay comparisons. These were placed on dry ice and transported to our laboratory, where the testes were dissected from the surrounding tissues, snap frozen in liquid nitrogen and stored at -80°C .

6.2.2 RNA extraction and cDNA synthesis

RNA extraction on ~50mg of testis from the 37 male dogs was performed as in section 2.2.3. The quality and quantity of RNA was measured using spectrophotometry as described in section 2.2.5. Initially, the smaller sections from each testis were placed in *RNAlater*®-ICE (Life Technologies, Paisley UK). This was to ease processing by maintaining RNA integrity if the tissue thawed. However, using this treatment resulted in unacceptably low 260/280 and 260/230 ratios. Therefore, RNA was extracted straight from the frozen tissue. This solved the quality issues, and all samples had 260/280 and 260/230 ratios over 2.0, but meant that the tissue had to be handled with great care to avoid RNA degradation.

cDNA synthesis was performed as per section 2.2.4. The quantity and quality of cDNA produced was measured using spectrophotometry, as detailed in section 2.2.5.

6.2.3 Global DNA methylation measurement

Percentage 5-methylcytosine (5-mc) was measured using an ELISA kit (Zymo Research, Tustin California USA). The kit was used to measure percentage 5-mc in leukocyte DNA from the study dogs with sufficient DNA remaining after telomere length analysis. This resulted in 41 dogs (36 male, 5 female) at time point 1 and 24 dogs (23 male, 1 female) at time point 2 being used for 5-mc analysis. Standard curves were run on each plate to quantify the percentage 5-mc in the test samples. All samples and standards were tested in duplicate and the mean absorbance readings of the two repeats taken.

The standard curve point samples were made by mixing the negative and positive controls provided in the kit. By combining different amounts of the positive and negative controls, the points on the standard curve were: 0%, 5%, 10%, 25%, 50%, 75% and 100% 5-mc. The volumes of the positive and negative controls were added to a 0.2ml microcentrifuge tube and brought to a final volume of 100µl with 5-mc coating buffer (kit provided). In addition, 100ng of each DNA sample was added to a 0.2ml microcentrifuge tube, then the volume made up to 100µl with 5-mc coating buffer. The tubes were placed in a thermal cycler and the DNA denatured at 98°C for 5 minutes. Immediately after, the tubes were put on ice for 10 minutes. The entire volume from the tubes was then added to the wells of a 96 well plate (kit provided). The plate was then covered in foil and placed in a 37°C incubator for one hour. Following the hour, the buffer was discarded from the wells, tapping out any remaining buffer. 200µl of 5-mc ELISA buffer (kit provided) was added to each well. The plate was recovered with foil and incubated at 37°C for a further 30 minutes. The buffer was then discarded from the wells. 100µl of an antibody mix, containing 1:2000 anti-5-mc and 1:1000 secondary antibody in 5-mc ELISA buffer, was then added to each well. The plate was again covered with foil, then incubated at 37°C for one hour. The antibody mix was then discarded, and the wells washed 3 times with 5-mc ELISA buffer. 100µl of horseradish peroxidase developer (kit provided) was added to each well. The colour was allowed to develop for 15 minutes, then absorbance was measured at 405nm using a plate reader (MTX Lab Systems, Virginia USA).

In order to calculate the percentage 5-mc in the test samples, the percentage 5-mc in the standards were plotted against their absorbance at 405nm. A curve was fitted to the points, and the line equation used to calculate the percentage 5-mc in the test samples from their absorbance at 405nm.

6.2.4 Data analysis

Linear modelling analyses were performed as per chapter 4. Pearson product correlation testing was used to assess relationships between mRNA expression levels. Spearman's rank correlation assessed the relationship between 5-mc at time points 1 and 2. Regression to the mean correction for the change in 5mc in relation to the baseline as per Chapter 5. Life expectancy corrected age was calculated as per Chapter 5.

6.3 Results

6.3.1 qRT-PCR optimisation

Primer sequences for CDKN2A and two reference genes, RPL13A and GAPDH, were kindly provided by Dr Dagmara McGuinness (Institute of Cancer Sciences, University of Glasgow). The remaining primers were designed using Primer Express 2.0 software (Applied Biosystems, Paisley UK). The design parameters were set as: optimum temperature 65°C (minimum 64°C, maximum 66°C), primer length optimum 20 bases (minimum 18, maximum 22) and amplicon length of between 160 and 200 base pairs. The primer sequences are shown in Table 6-1.

Table 6-1 Primer sequences for mRNA expression qRT-PCR assays

Gene	Gene name	Genbank number	Forward primer / reverse primer
RPL13A	Ribosomal protein L13A	AJ388525	GCCGGAAGGTTGTAGTCGT / GGAGGAAGGCCAGGTAATTC
GAPDH	Glyceraldehyde -3-phosphate dehydrogenase	AB038240	TCACCAGGGCTGCTTTTAAC / AGGAGGCATTGCTGACAATC
CDKN2A	Cyclin-dependent kinase inhibitor 2A	AB675384	GGTCGGAGCCCGATTCA / ACGGGGTCGGCACAGTT
3BHSD	3B-hydroxysteroid dehydrogenase	NM1010954	CCCAACTCCTACAGGGACATCATCC / GCACAAGTATGCAAAGTGCCACCA
17BHSD	17B-hydroxysteroid dehydrogenase	XM3638870	CCAAACCTCCTCCCGAGCCA / AGGGCCACCCCGGAAGAAAT
AHR	Aryl hydrocarbon receptor	XM532485	TGTCTGCTGGATAATTCGTCCGG / TCAAGGATGGATGGTGGTTGAAGTG
CLU	Clusterin	NM1003370	GGCTAGTTGGCCACCAGCTCG / CATGATGCTGGATGCCCCGGTT
P450SCC	Cytochrome P450 side-chain-cleaving	XM535539	AGACCCGTCGGCGTCCTGTT / TGCTTGATGCGCCTGTGCAG
SIRT1	Sirtuin 1	XP546130	CAATTGAAGATGCTGTGAAATTACTGCAA / TGCTTGAGGATCTGGAAGGTCTGG
StAR	Steroidogenic acute regulatory protein	EF522840	AGGTGAGCATGGTCCCACGTG / CGCAGGTGGTTGGCAAAATCC

TERT	Telomerase reverse transcriptase	NM1031630	GGCTGGTAGAGGTGATTGCCAATG/ AAACTGTCTCATGTAAGGCTGCAGGTCT
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CDKN2A mRNA expression levels were measured first and separately to the other genes. The primer concentrations were optimised for the target gene and two reference genes. As in chapter 4, an optimisation matrix of primer concentrations was used to check several combinations of forward and reverse primers. 25ng of cDNA made from the testes obtained from the SSPCA were added to the positive control wells and ddH₂O to the blank wells. 10µl of Absolute blue® SYBR green low ROX mix was added to each primer combination, cDNA if appropriate and ddH₂O added to a final volume of 20µl per well. The resultant forward/reverse primer concentrations were RPL13A 100/150nM, GAPDH 100/100nM and CDKN2A 100/100nM. However, it was noticed that the C_Ts of the CDKN2A PCRs were very high with 25ng of cDNA, likely due to the low expression of the gene in the testis. A range of cDNA amounts were tested to see what amount would result in acceptable C_T values in the target and reference genes. As a result, 250ng was added to each well in subsequent experiments.

All three PCRs began with a 95°C 15 minute incubation to activate the Thermo-Start™ DNA polymerase. The thermal cycling profile then consisted of 95°C for 15 seconds, 53°C for 30 seconds and 72°C for 30 seconds. The thermal cycling was run for 45 cycles in the CDKN2A PCR, and 40 cycles in the RPL13A and GAPDH PCRs, as the average C_T value was higher in the CDKN2A PCR than in the other PCRs. Following the cycles, melt curves were run for all PCRs (60-95°C). All the PCRs were performed on an Applied Biosystems 7500fast machine (Applied Biosystems, Paisley UK) using machine compatible white 96 well plates (Starlab, Milton Keynes UK). Mastermixes were made for the PCR set ups, 15µl of which were added to each well, followed by 5µl of test sample, ddH₂O for no template control wells, or, on the initial run, 5µl of control with no reverse transcriptase added. This control

was generated from a mix of RNA samples, and put through the cDNA synthesis procedure, but instead of reverse transcriptase, ddH₂O was added instead. By including this control, it can be seen if contaminating genomic DNA is present, which was not removed during the on-column DNase treatment. It is highly unlikely that all DNA would have been removed, but by running this control, it can be seen to what extent contaminating genomic DNA contributes to amplification in the test wells. For example, if the no reverse transcriptase wells amplify 10 cycles later than the test wells, the control wells contain roughly one thousandth of target sequence compared to test wells (based on 100% PCR efficiency), and 0.1% of amplification in the test wells is due to genomic DNA presence. The plates were sealed with Microamp optical cap strips (Applied Biosystems, Paisley UK) and briefly centrifuged. The plates were protected from light where possible, as SYBR green is light sensitive. DNAZap® was used prior to setting up experiments to clean surfaces and pipettes, and filter pipette tips were used.

The relative expression of CDKN2A was calculated using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). This method measures the expression of the gene relative to the expression in a control sample. The sample from the testis of the 1 year-old obtained from the SSPCA was used as this control. The gene of interest expression is also corrected for the expression of a housekeeping gene, which corrects for different amounts of cDNA being loaded into the wells. In this case, the relative expression was calculated twice, using each housekeeping gene in turn. The formula for calculation is as follows:

$$\begin{aligned} & \text{relative expression} \\ &= 2^{-[(C_t^{CDKN2A \text{ sample}} - C_t^{\text{Housekeeping sample}}) - (C_t^{CDKN2A \text{ control}} - C_t^{\text{Housekeeping control}})]} \end{aligned}$$

Two housekeeping genes were used as previously housekeeping genes have been shown to vary in expression so using more than one gene may help with errors related to this (Vandesompele *et al.*, 2002). The mean of relative expressions using each housekeeping gene was taken.

Each sample and control was run in triplicate. The mean C_T value from the three wells was used for analysis. As with the telomere assay, if the percentage coefficient of variation for the three wells was 5 or above, the sample was repeated. The sample from the 3 year-old from the SSPCA was run on each plate to calculate the inter-assay coefficient of variation, which was 4.6%.

Subsequently, the assays for the remaining genes were optimised and performed. The conditions for the housekeeping genes were kept the same. On advice, to save time and money, the primers were all checked to see if they would operate at 100nM/100nM concentrations. Each pair was run with 100ng cDNA, ddH₂O and no reverse transcriptase control samples. 100nM concentrations for all primers was found to be acceptable. In addition, as 100ng cDNA produced acceptable C_T values, this amount of cDNA was used for all reactions. Due to machine availability, these PCRs were performed on a MxPro 3000P qPCR machine (San Diego, California USA) using machine compatible 96 well plates (Thermo Scientific, Paisley UK). The gene of interest PCRs began with a 15-minute incubation, followed by 45 cycles of 95°C for 25 seconds, 63°C for 25 seconds and 72°C for 25 seconds, and finally a melt curve (63-95°C). As with previous experiments, the plates were set up using 15µl of mastermix and 5µl of sample or ddH₂O. Plate seals were adhered to the plate and then the plate briefly centrifuged. The relative expressions were calculated as per CDKN2A expression.

The median and interquartile range of relative mRNA expression for each gene are presented in Table 6-2.

Table 6-2 Relative mRNA expression levels

Median and interquartile range of mRNA expression levels of tested genes relative to a control sample

Gene	Median expression	Interquartile range
3 beta HSD	0.70	0.61
17 beta HSD	0.58	0.22
AHR	0.74	0.37
CDKN2A	0.36	0.36
Clusterin	1.28	1.45
P450 SCC	0.41	0.37
Sirtuin 1	0.59	0.47
StAR	0.91	0.62
TERT	0.60	0.47

Following Shapiro-Wilk tests, 3BHSD, 17BHSD, AHR, p450 SCC and CDKN2A expression levels were natural log transformed and the square root of sirtuin 1, StAR and TERT expression levels were taken to normalise the distributions of the data. Values greater than two standard deviations greater or less than the mean were treated as outliers and excluded.

The interindividual Pearson correlation coefficients between pairs of mRNA expression levels are presented in Table 6-3. CDKN2A and 17BHSD expression levels were significantly negatively correlated ($p=0.01$). 17BHSD and sirtuin 1 were significantly positively correlated ($p=0.02$). Clusterin expression was significantly positively correlated with sirtuin 1 ($p=0.01$) expression levels. P450 SCC expression levels were significantly positively correlated to StAR ($p=0.02$) and TERT ($p=0.02$) expression levels.

Table 6-3 Correlations between mRNA gene expression levels

Correlation coefficients of pairwise comparisons between mRNA gene expression levels. Significance levels are donated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

ln 3 beta	-0.001							
ln 17 beta	-0.40*	0.13						
ln AHR	-0.27	0.14	0.20					
Clusterin	0.17	-0.09	-0.09	-0.17				
ln p450								
SCC	0.15	0.27	-0.05	-0.002	-0.007			
sqrt Sirt1	0.07	0.07	0.39*	0.29	0.40*	0.10		
sqrt StAR	-0.07	-0.23	0.13	-0.15	0.30	0.38*	0.13	
sqrt TERT	0.18	-0.30	-0.14	-0.006	0.33*	0.37*	0.32	0.14
	ln CDKN2A	ln 3βHSD	ln 17βHSD	ln AHR	Clusterin	ln p450 SCC	sqrt Sirt1	sqrt StAR

6.3.2 Age

3BHSD mRNA expression significantly increased with age in a linear model ($F=30.81_{(1,35)}$, $R^2=0.47$, $p<0.001$). When the “poly” function was employed, the fit of age to 3BHSD model was improved ($F=20.65_{(2,34)}$, $R^2=0.55$, $p<0.001$, Figure 6.1A). 3BHSD mRNA expression also increased with increasing life expectancy (LE) adjusted age ($F=36.02_{(1,26)}$, $R^2=0.58$, $p<0.001$, Figure 6.1B). The fit of this model, however, was better not using a polynomial variable. Linear models of age and LE adjusted age to 17BHSD were not statistically significant (Age: $F=2.28_{(1,34)}$, $R^2=0.06$, $p=0.14$; LE adjusted age: $F=3.96_{(1,25)}$, $R^2=0.14$, $p=0.06$, Figure 6.1C&D). The linear models of age and LE adjusted age to p450 SCC were not statistically significant (age: $F=1.00_{(1,33)}$, $R^2=0.03$, $p=0.33$; LE adjusted age: $F=0.02_{(1,24)}$, $R^2<0.001$, $p=0.90$, Figure 6.2A&B). Similarly, the linear models of age and LE adjusted age to StAR were not statistically significant (age: $F=1.77_{(1,33)}$, $R^2=0.05$, $p=0.19$; LE adjusted age: $F=3.44_{(1,23)}$, $R^2=0.13$, $p=0.08$, Figure 6.2C&D).

Clusterin mRNA expression decreased non-significantly with age (AIC=87.52, $p=0.07$, Figure 6.3A). Life expectancy adjusted age significantly decreased with increasing clusterin mRNA expression (AIC=63.41, $p<0.001$, Figure 6.3B). Age and LE adjusted age non-significantly decreased with increasing CDKN2A mRNA expression level (age: $F=1.57_{(1,35)}$, $R^2=0.04$, $p=0.22$; LE adjusted age: $F=1.24_{(1,25)}$, $R^2=0.05$, $p=0.28$, Figure 6.3C&D). The linear models of age and LE adjusted age to sirtuin 1 mRNA expression level were not statistically significant (age: $F=0.73_{(1,34)}$, $R^2=0.02$, $p=0.40$; LE adjusted age: $F=2.53_{(1,24)}$, $R^2=0.10$, $p=0.12$). In addition, it appeared that a polynomial regression may be a better model. The model of age² to sirtuin 1 mRNA expression level neared significance ($F=3.83_{(1,34)}$, $R^2=0.10$, $p=0.06$, Figure 6.4A). In addition, using polynomial regression, the model of LE adjusted age to sirtuin 1 was highly significant ($F_{(2,24)}=138.47$, $R^2=0.92$, $p<0.001$, Figure 6.4B).

Age was not significantly related to TERT mRNA expression level ($F=0.45_{(1,33)}$, $R^2=0.01$, $p=0.51$, Figure 6.4C). The linear model of LE adjusted age to TERT mRNA expression was not statistically significant ($F=0.006_{(1,23)}$, $R^2<0.001$, $p=0.94$, Figure 6.4D). The linear model of age to AHR mRNA expression was not statistically significant ($F=0.04_{(1,33)}$, $R^2=0.001$, $p=0.84$, Figure 6.5A). Although, the LE adjusted age significantly increased with AHR mRNA expression level ($F=20.14_{(1,25)}$, $R^2=0.45$, $p<0.001$, Figure 6.5B).

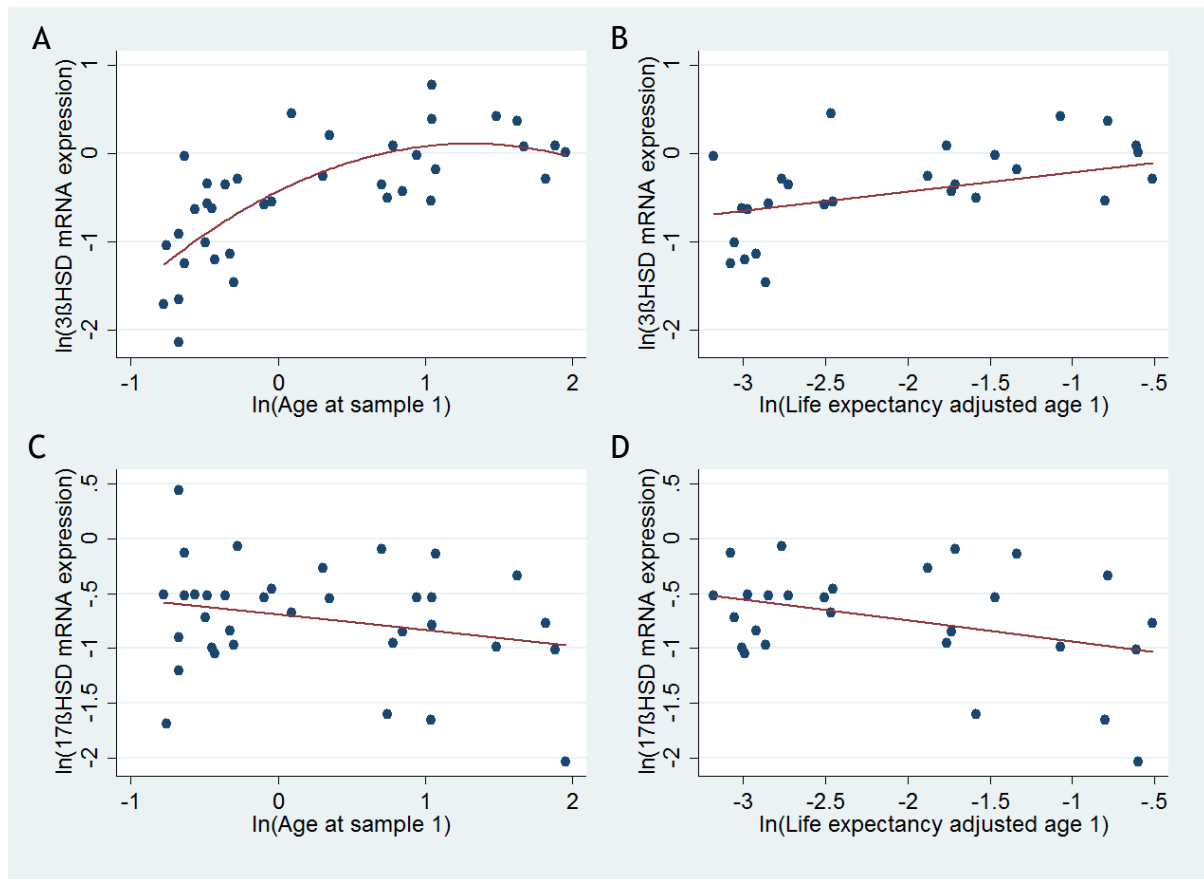


Figure 6.1 3βHSD & 17βHSD gene expression levels by dog age

Relative mRNA expression of 3βHSD with dog age (A, N=37, $R^2=0.55$, $p<0.001$) and life-expectancy adjusted age (B, N=27, $R^2=0.58$, $p<0.001$); and 17βHSD relative expression with dog age (C, N=36, $R^2=0.06$, $p=0.14$) and life-expectancy adjusted age (D, N=27, $R^2=0.14$, $p=0.06$)

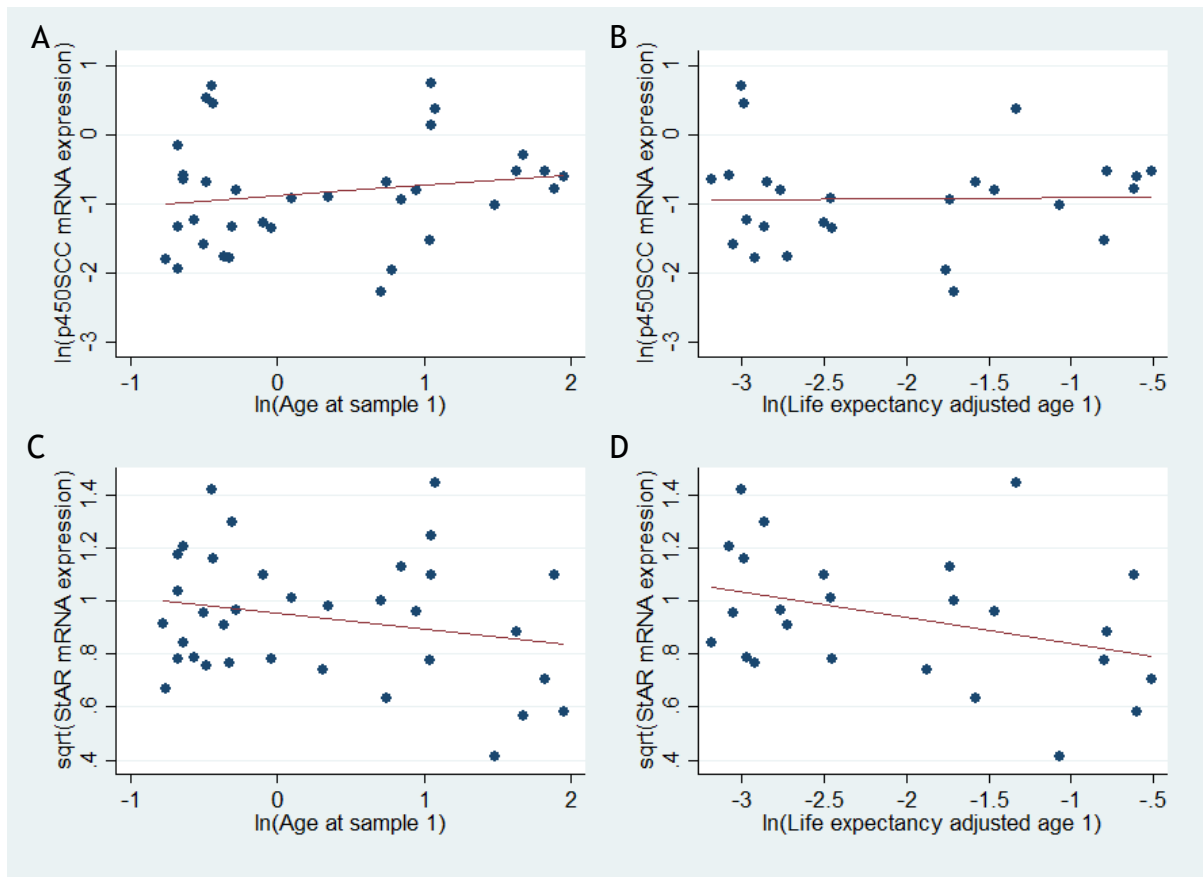


Figure 6.2 p450SCC & StAR gene expression levels by dog age

Relative mRNA expression of p450 SCC with dog age (A, $N=35$, $R^2=0.03$, $p=0.33$) and life-expectancy adjusted age (B, $N=26$, $R^2<0.001$, $p=0.90$); and StAR relative expression with dog age (C, $N=35$, $R^2=0.05$, $p=0.19$) and life-expectancy adjusted age (D, $N=25$, $R^2=0.13$, $p=0.08$)

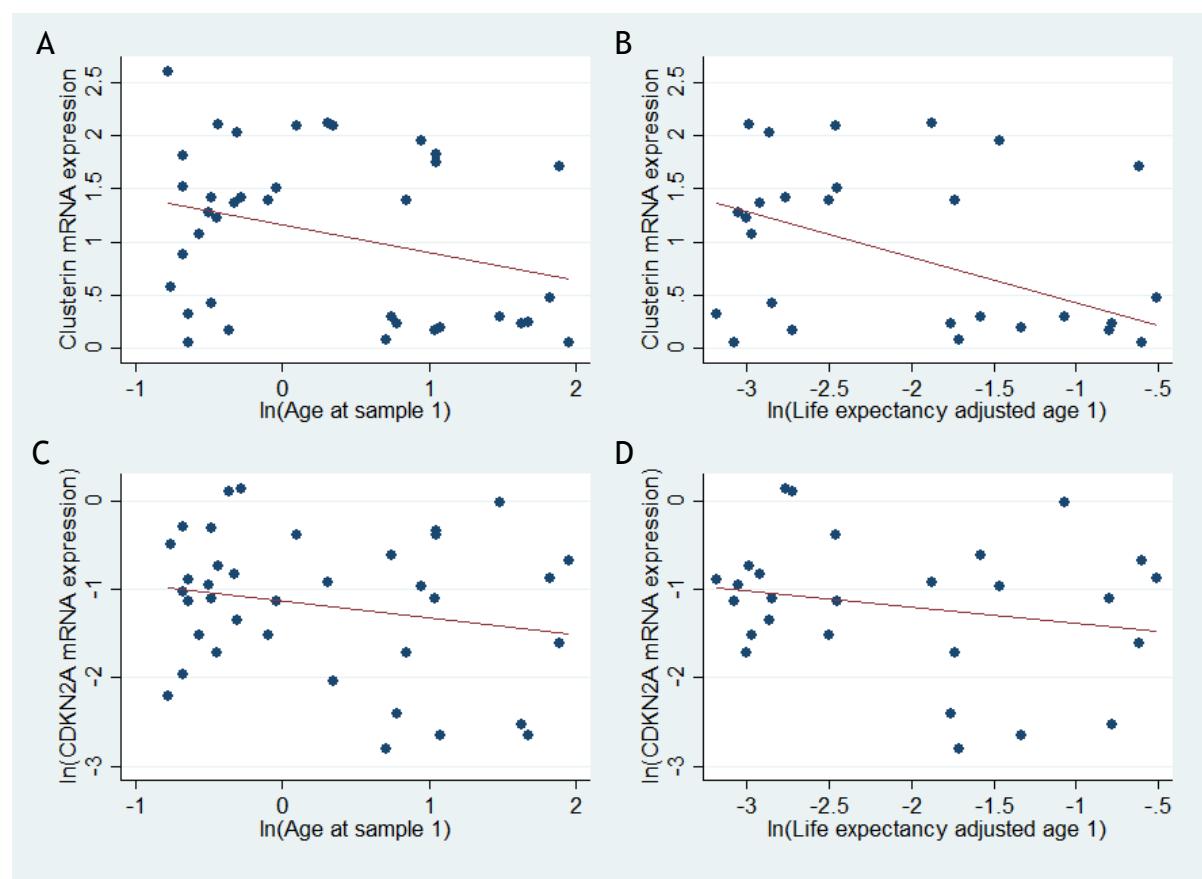


Figure 6.3 Clusterin & CDKN2A gene expression levels by dog age

Relative mRNA expression of clusterin with dog age (A, N=37, AIC=87.52, $p=0.07$) and life-expectancy adjusted age (B, N=27, AIC=63.41, $p<0.001$); and CDKN2A relative expression with dog age (C, N=37, $R^2=0.04$, $p=0.22$) and life-expectancy adjusted age (D, N=27, $R^2=0.05$, $p=0.28$)

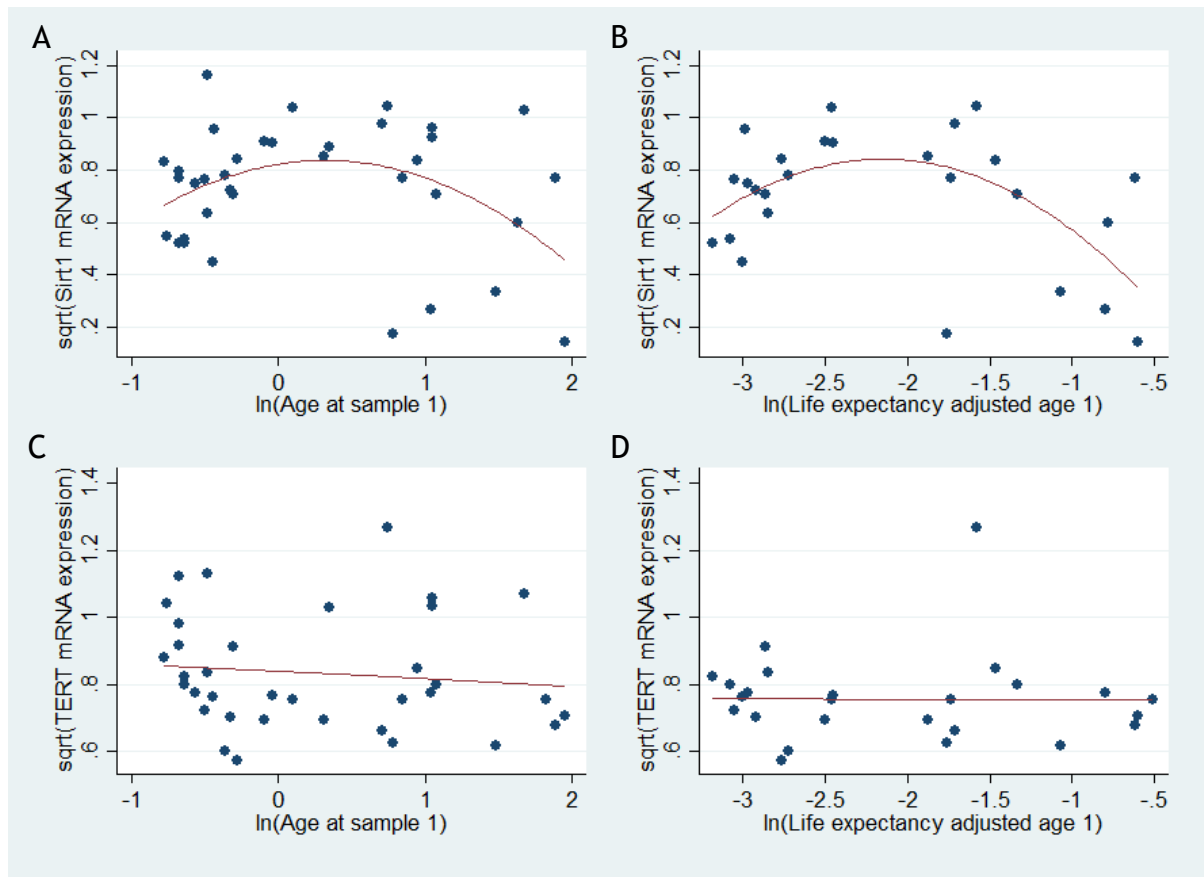


Figure 6.4 Sirt1 & TERT gene expression levels by dog age

Relative mRNA expression of sirtuin 1 with dog age (A, $N=36$, $R^2=0.10$, $p=0.06$) and life-expectancy adjusted age (B, $N=27$, $R^2=0.92$, $p<0.001$); and TERT relative expression with dog age (C, $N=35$, $R^2=0.01$, $p=0.51$) and life-expectancy adjusted age (D, $N=25$, $R^2<0.001$, $p=0.94$)

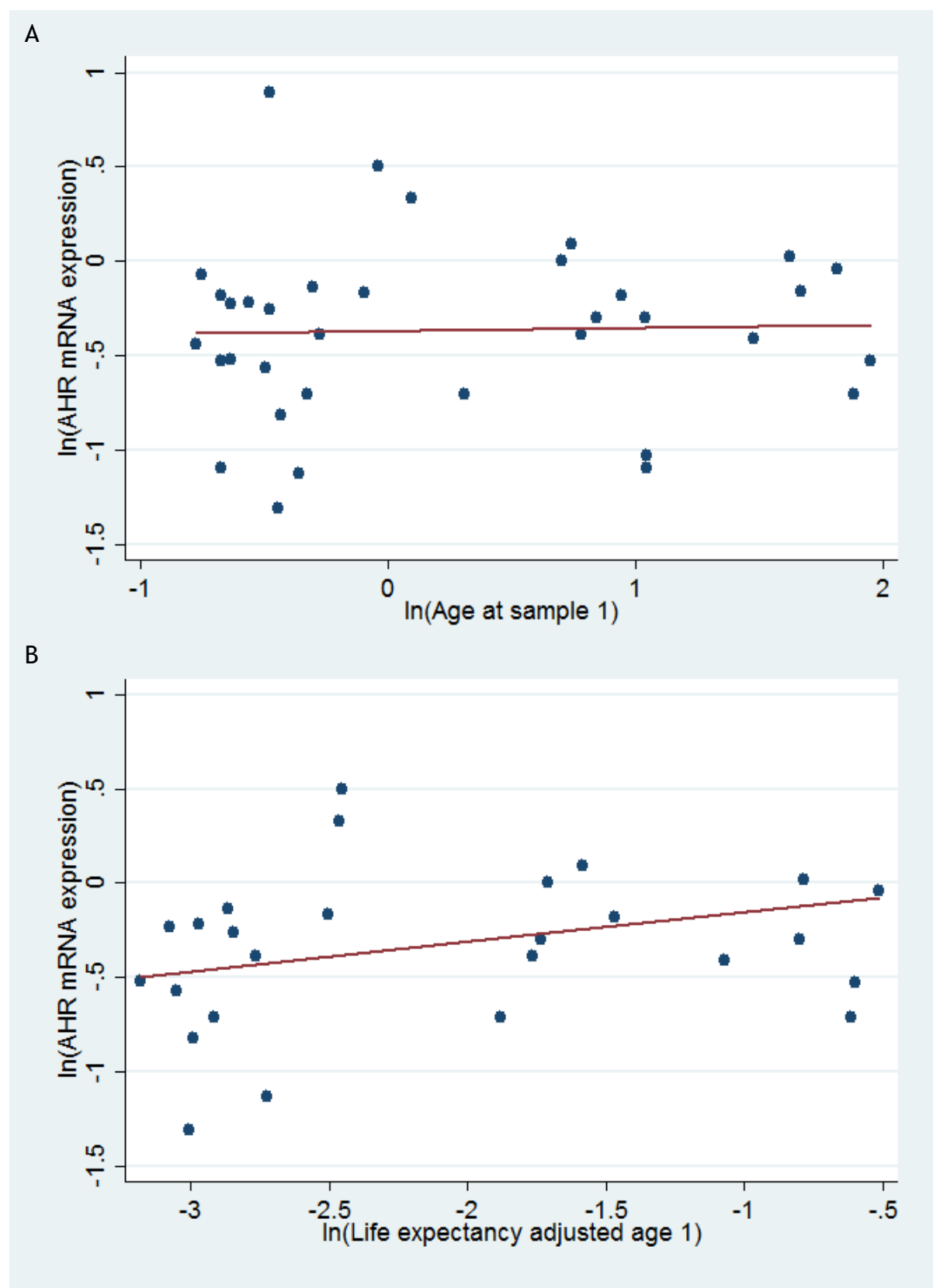


Figure 6.5 AHR gene expression levels by dog age

Relative mRNA expression of AHR with dog age (A, $N=35$, $R^2=0.001$, $p=0.84$) and life-expectancy adjusted age (B, $N=27$, $R^2=0.45$, $p<0.001$)

6.3.3 Nicotine

3BHSD mRNA expression non-significantly increased with increasing HNC1 ($F=2.31_{(1,35)}$, $R^2=0.06$, $p=0.14$, Figure 6.6A). There was a non-significant decrease in 17BHSD mRNA expression with increasing HNC1 ($F=1.18_{(1,34)}$, $R^2=0.03$, $p=0.29$, Figure 6.6C). The linear model of HNC1 to p450 SCC mRNA expression level was not statistically significant ($F<0.001_{(1,33)}$, $R^2<0.001$, $p=0.94$, Figure 6.7A). Similarly, the linear model of HNC1 to StAR mRNA expression was not significant ($F=0.85_{(1,33)}$, $R^2=0.03$, $p=0.36$, Figure 6.7C).

There was a non-significant decrease in clusterin mRNA expression with increasing HNC ($AIC=89.75$, $p=0.29$, Figure 6.8A). CDKN2A mRNA expression significantly increased with HNC1 ($F=9.87_{(1,35)}$, $R^2=0.22$, $p=0.003$, Figure 6.8C). The linear model of HNC to sirtuin1 mRNA expression was not statistically significant ($F=0.21_{(1,34)}$, $R^2=0.006$, $p=0.65$, Figure 6.9A). The linear model of HNC1 to TERT mRNA expression was not statistically significant ($F=0.66_{(1,33)}$, $R^2=0.02$, $p=0.42$, Figure 6.9C). The linear model of HNC1 to AHR mRNA expression level was not significant ($F=0.06_{(1,33)}$, $R^2=0.002$, $p=0.81$, Figure 6.10A).

6.3.4 Cotinine

There was a non-significant increase in 3BHSD mRNA expression level with HCC1 ($F=3.27_{(1,35)}$, $R^2=0.09$, $p=0.08$, Figure 6.6B). Whereas, 17BHSD mRNA expression level non-significantly decreased with HCC ($F=1.55_{(1,34)}$, $R^2=0.04$, $p=0.22$, Figure 6.6D). The linear model of HCC1 to p450 SCC mRNA expression level was not statistically significant ($F=0.003_{(1,33)}$, $R^2<0.001$, $p=0.95$, Figure 6.7B). There was a non-significant decrease in StAR mRNA expression with increasing HCC1 ($F=1.23_{(1,33)}$, $R^2=0.04$, $p=0.27$, Figure 6.7D).

Clusterin mRNA expression significantly decreased with increasing HCC1 ($AIC=86.16$, $p=0.03$, Figure 6.8B). CDKN2A mRNA significantly increased with

increasing HCC1 ($F=7.39_{(1,35)}$, $R^2=0.17$, $p=0.01$, Figure 6.8D). The linear model of HCC1 to sirtuin 1 mRNA expression level was not statistically significant ($F=0.92_{(1,34)}$, $R^2=0.03$, $p=0.34$, Figure 6.9B). TERT mRNA expression level was not related to HCC1 (Figure 6.9D, $F=0.51_{(1,33)}$, $R^2=0.02$, $p=0.48$). There was no statistically significant relationship between HCC1 and AHR mRNA expression level ($F=0.01_{(1,33)}$, $R^2<0.001$, $p=0.91$, Figure 6.10B).

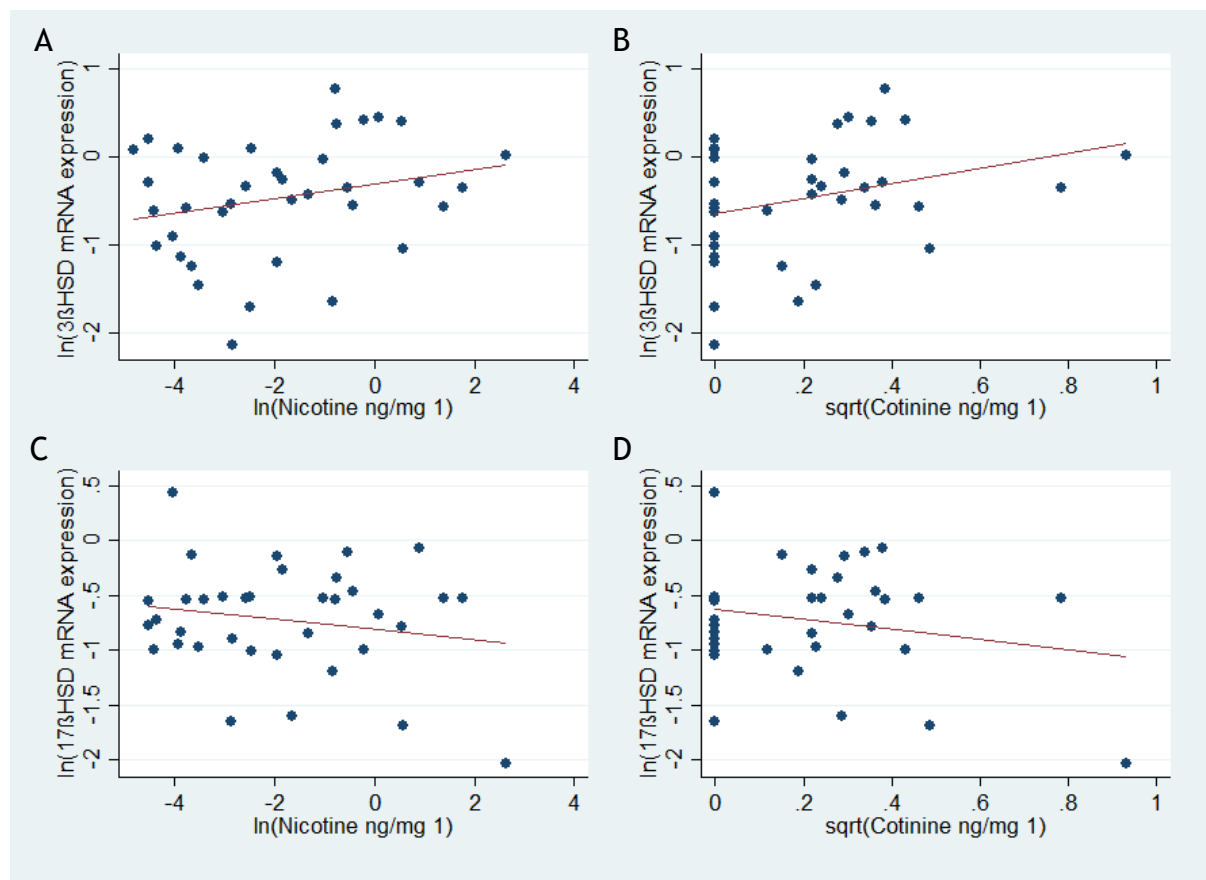


Figure 6.6 $3\beta\text{HSD}$ & $17\beta\text{HSD}$ gene expression levels and ETS biomarker concentrations

Relative mRNA expression of $3\beta\text{HSD}$ with hair nicotine concentration (A, $N=37$, $R^2=0.06$, $p=0.14$) and hair cotinine concentration (B, $N=37$, $R^2=0.09$, $p=0.08$); and $17\beta\text{HSD}$ relative expression with hair nicotine concentration (C, $N=36$, $R^2=0.03$, $p=0.29$) and hair cotinine concentration (D, $N=36$, $R^2=0.04$, $p=0.22$)

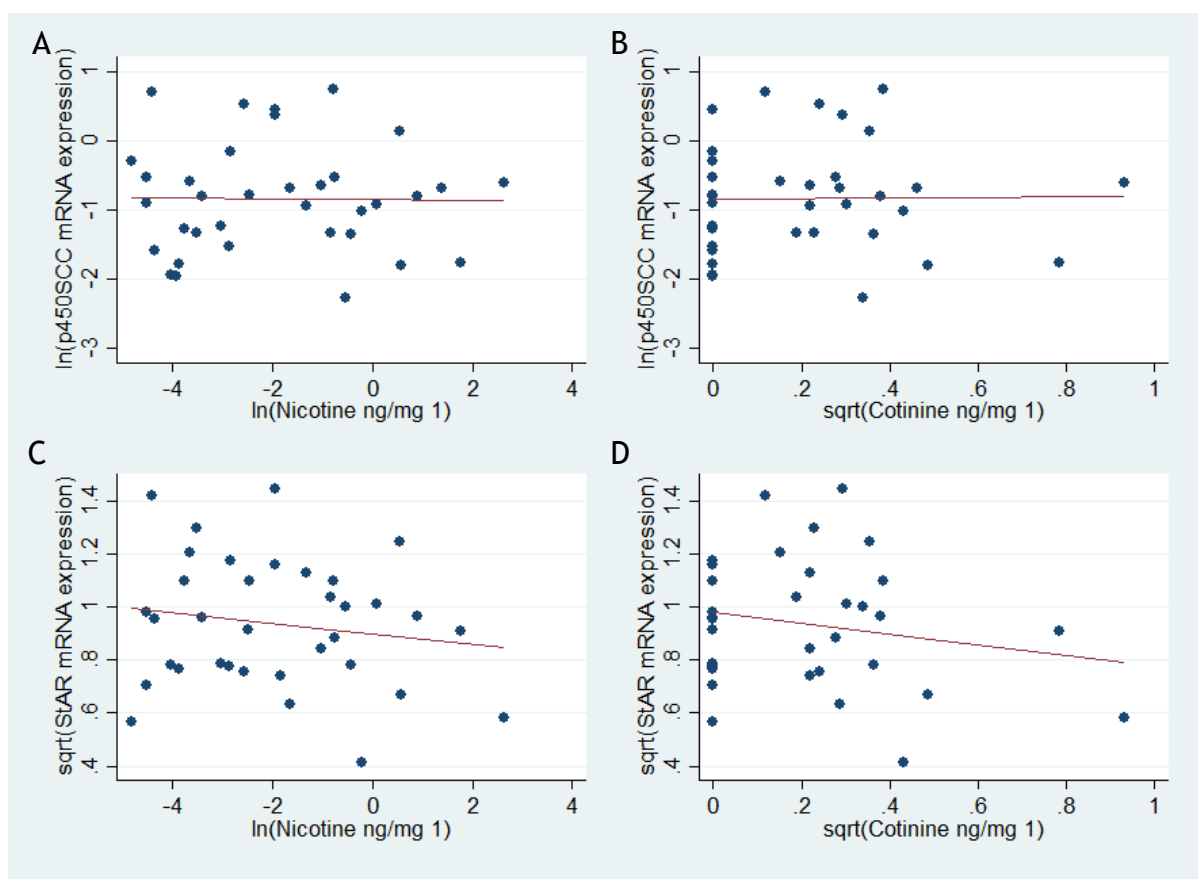


Figure 6.7 p450SCC & StAR gene expression levels and ETS biomarker concentrations

Relative mRNA expression of p450 SCC with hair nicotine concentration (A, N=35, $R^2 < 0.001$, $p = 0.94$) and hair cotinine concentration (B, N=35, $R^2 < 0.001$, $p = 0.95$); and StAR relative expression with hair nicotine concentration (C, N=35, $R^2 = 0.03$, $p = 0.36$) and hair cotinine concentration (D, N=35, $R^2 = 0.04$, $p = 0.27$)

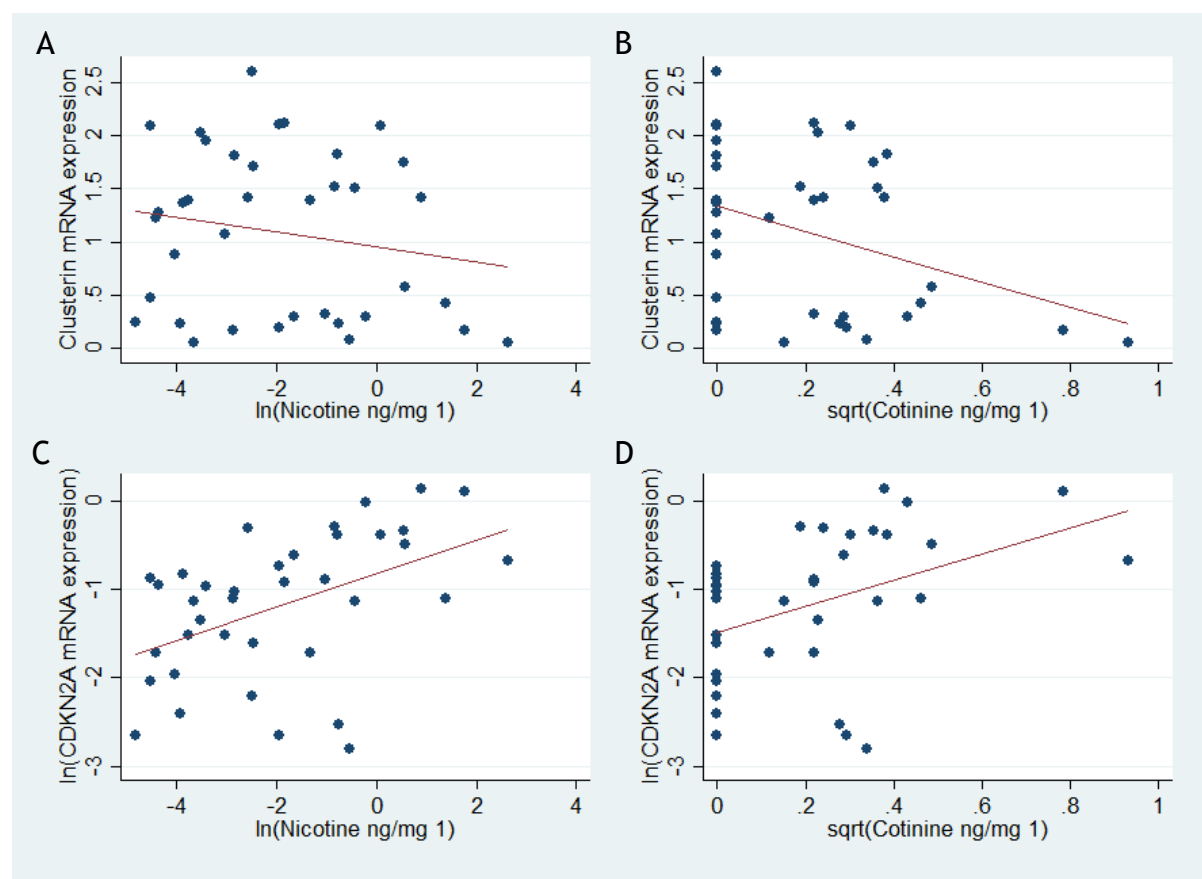


Figure 6.8 Clusterin & CDKN2A gene expression levels and ETS biomarker concentrations

Relative mRNA expression of clusterin with hair nicotine concentration (A, $N=37$, $AIC=89.75$, $p=0.29$) and hair cotinine concentration (B, $N=37$, $AIC=86.16$, $p=0.03$); and CDKN2A relative expression with hair nicotine concentration (C, $N=37$, $R^2=0.22$, $p=0.03$) and hair cotinine concentration (D, $N=37$, $R^2=0.17$, $p=0.01$)

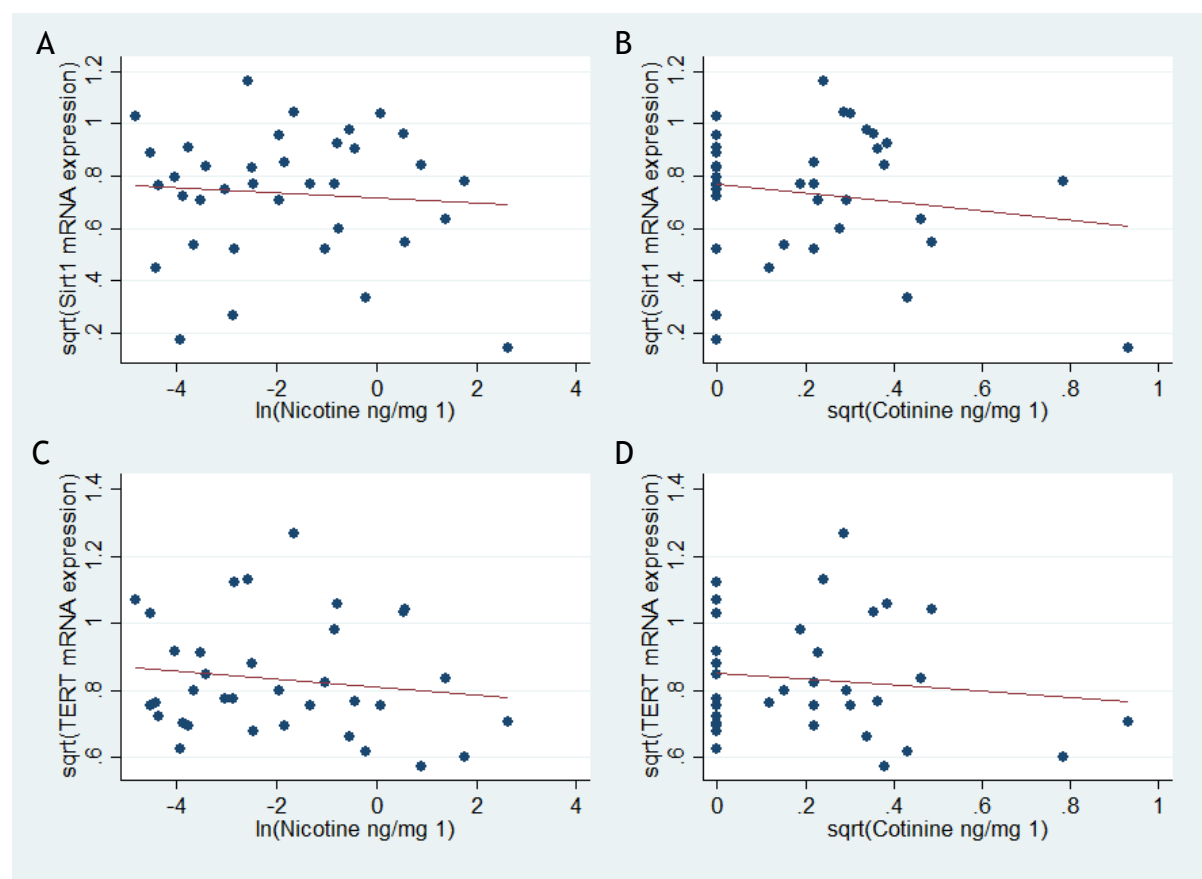


Figure 6.9 Sirt1 & TERT gene expression levels and ETS biomarker concentrations

Relative mRNA expression of sirtuin 1 with hair nicotine concentration (A, $N=36$, $R^2=0.006$, $p=0.65$) and hair cotinine concentration (B, $N=36$, $R^2=0.03$, $p=0.34$); and TERT relative expression with hair nicotine concentration (C, $N=35$, $R^2=0.02$, $p=0.42$) and hair cotinine concentration (D, $N=35$, $R^2=0.02$, $p=0.48$)

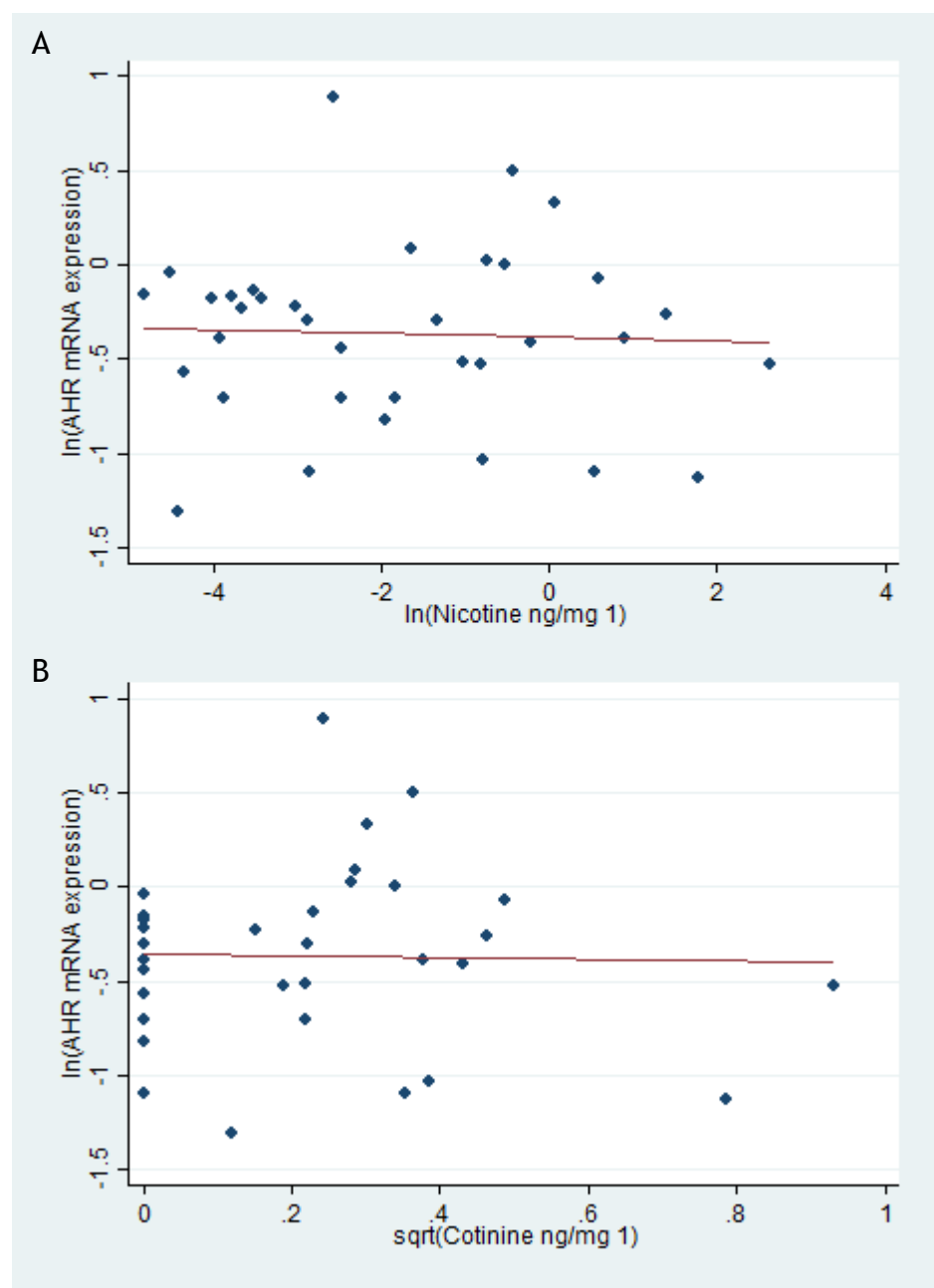


Figure 6.10 AHR gene expression levels and ETS biomarker concentrations 5

Relative mRNA expression of AHR with hair nicotine concentration (A, N=35, $R^2=0.002$, $p=0.81$) and hair cotinine concentration (B, N=35, $R^2<0.001$, $p=0.91$)

6.3.5 Further analyses

Dog weight was statistically significantly modelled to all gene mRNA expression levels except p450 SCC and TERT (Figures 6.11-6.13), with weight being negatively related to CDKN2A mRNA ($F=80.81_{(1,36)}$, $R^2=0.69$, $p<0.001$), 3BHSD mRNA ($F=14.67_{(1,36)}$, $R^2=0.29$, $p<0.001$), 17BHSD mRNA ($F=67.04_{(1,35)}$, $R^2=0.66$, $p<0.001$) and AHR mRNA ($F=22.08_{(1,34)}$, $R^2=0.39$, $p<0.001$) expression levels, and weight being positively associated to clusterin mRNA ($AIC=83.30$, $p<0.001$), sirtuin 1 mRNA ($F=339.5_{(1,35)}$, $R^2=0.91$, $p<0.001$) and StAR mRNA ($F=4.46_{(1,33)}$, $R^2=0.12$, $p=0.04$) expression levels. Weight was also non-significantly positively related to p450 SCC mRNA ($F=2.92_{(1,33)}$, $R^2=0.08$, $p=0.10$) and TERT mRNA ($F=1.31_{(1,33)}$, $R^2=0.04$, $p=0.26$) expression levels.

Breed life expectancy was also significantly related to 8 of the 9 gene mRNA expression levels. Life expectancy was significantly negatively related to CDKN2A mRNA ($F=57.81_{(1,26)}$, $R^2=0.69$, $p<0.001$), 3BHSD mRNA ($F=16.97_{(1,26)}$, $R^2=0.40$, $p<0.001$), 17BHSD mRNA ($F=59.17_{(1,26)}$, $R^2=0.69$, $p<0.001$), AHR mRNA ($F=20.52_{(1,25)}$, $R^2=0.45$, $p<0.001$) and p450 SCC mRNA expression ($F=39.47_{(1,25)}$, $R^2=0.61$, $p<0.001$) levels. Whereas, life expectancy was positively associated with clusterin mRNA ($AIC=63.04$, $p<0.001$), sirtuin 1 mRNA ($F=167.90_{(1,25)}$, $R^2=0.87$, $p<0.001$) and StAR mRNA ($F=344.10_{(1,24)}$, $R^2=0.93$, $p<0.001$) levels. The linear model of life expectancy to TERT mRNA levels was not statistically significant ($F=0.77_{(1,23)}$, $R^2=0.03$, $p=0.39$).

Rudimentary analyses were carried out to see if any breed differences were evident in the mRNA expression levels of any of the genes. A paired t-test showed that 17BHSD mRNA expression levels ($p=0.001$) and AHR mRNA expression levels ($p=0.008$) were significantly higher in Staffordshire bull terriers than all other dogs. Whereas, Staffordshire bull terriers had significantly lower CDKN2A expression levels ($p=0.03$) than all other dogs. When Staffordshire bull terrier or not was included in a linear model with HNC to CDKN2A expression, the model was significant, with Staffordshire bull terriers having a decrease in CDKN2A with HNC

unlike other dogs ($F=15.64_{(3,32)}$, $R^2=0.59$, $p<0.001$). Clusterin mRNA and TERT mRNA expression levels were significantly lower in pure bred dogs than cross breeds ($p=0.02$ and $p<0.001$, respectively). No significant differences between breeds were found in the remaining genes.

6.3.6 Protein level of CDKN2A

A subset of samples had protein levels of CDKN2A to examine if the mRNA expression level correlated with protein level. The methods, optimisation process and results are detailed in Appendix 11.

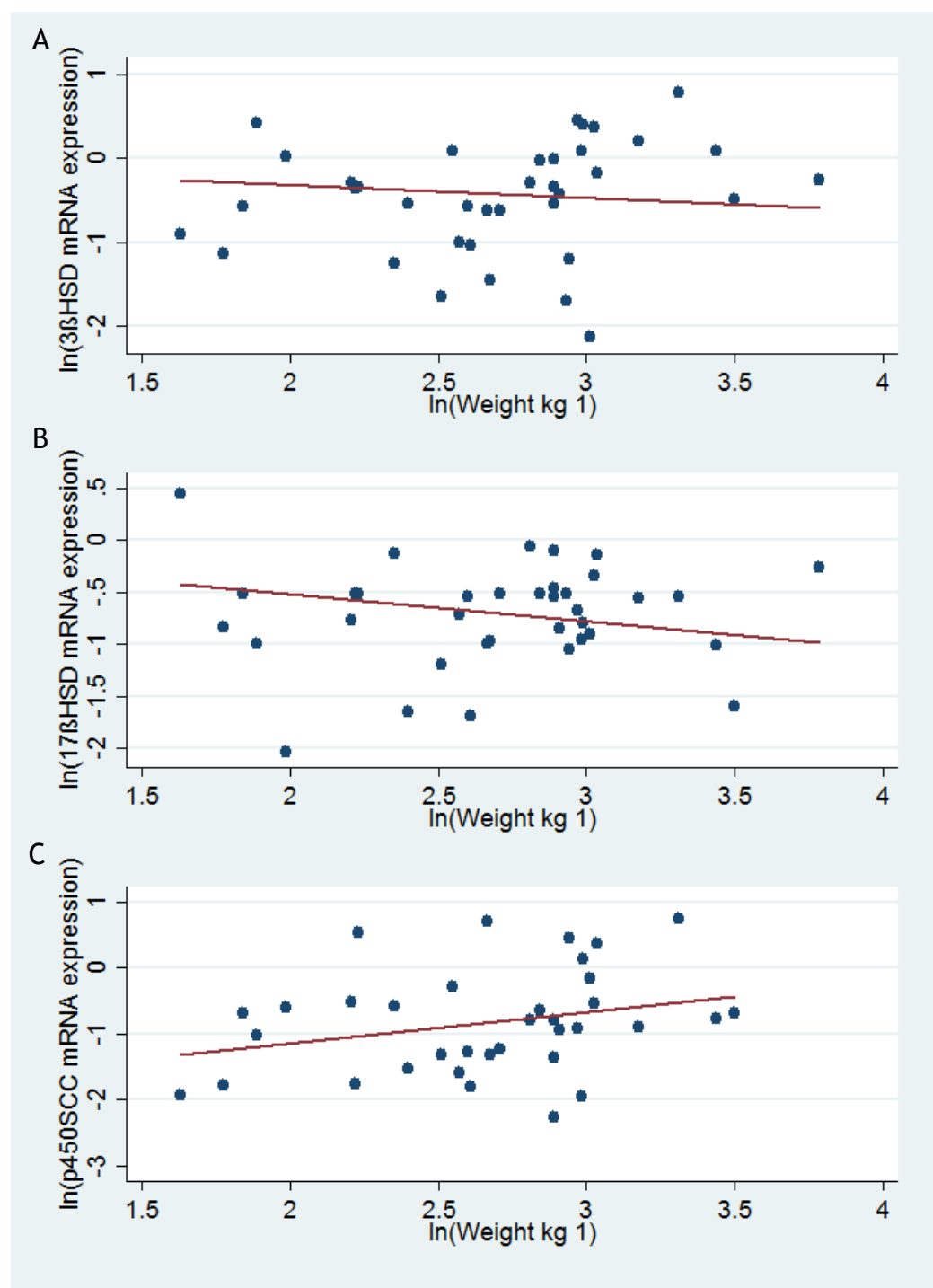


Figure 6.11 3βHSD, 17βHSD & p450SCC gene expression levels and dog weight
 Dog weight with relative mRNA expression of 3βHSD (A, N=37, $R^2=0.29$, $p<0.001$), 17βHSD (B, N=36, $R^2=0.66$, $p<0.001$) and p450 SCC (C, N=35, $R^2=0.08$, $p=0.10$)

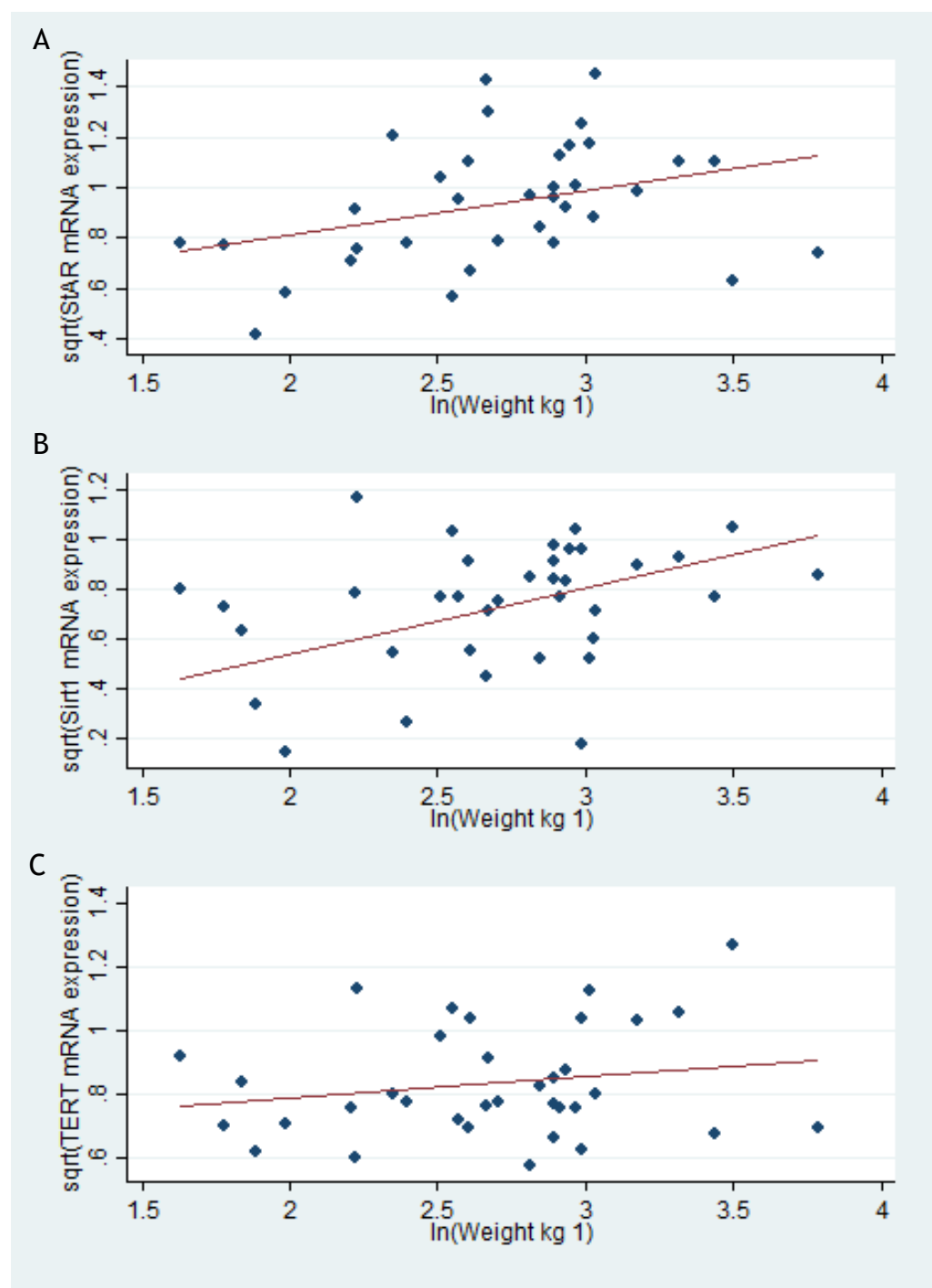


Figure 6.12 StAR, sirt1 & TERT gene expression levels and dog weight

Dog weight with relative mRNA expression of StAR (A, N=35, $R^2=0.12$, $p=0.04$), sirtuin 1 (B, N=36, $R^2=0.91$, $p<0.001$) and TERT (C, N=35, $R^2=0.04$, $p=0.26$)

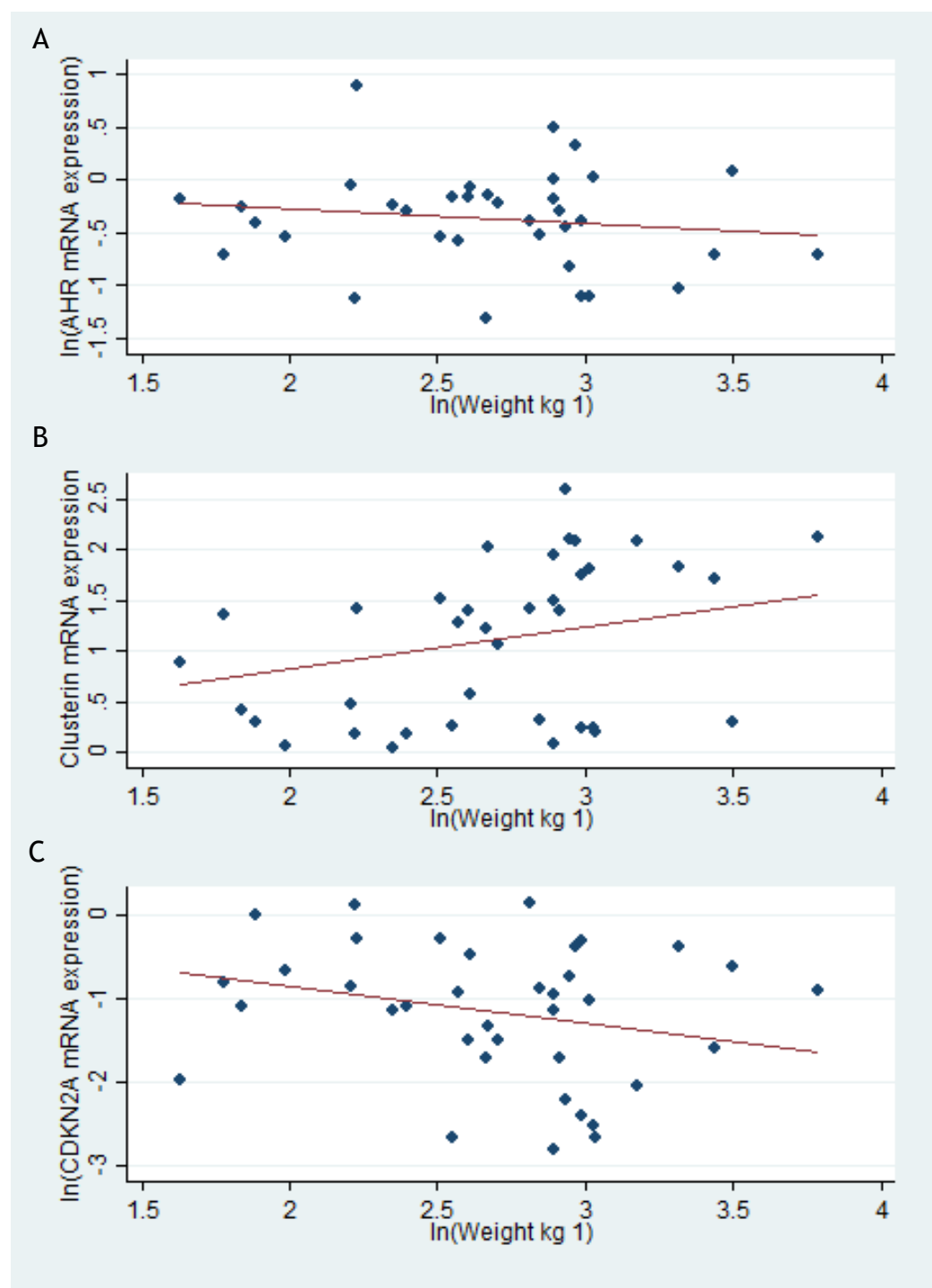


Figure 6.13 AHR, clusterin & CDKN2A gene expression levels and dog weight

Dog weight with relative mRNA expression of AHR (A, N=35, $R^2=0.39$, $p<0.001$), clusterin (B, N=37, AIC=83.30, $p<0.001$) and CDKN2A (C, N=37, $R^2=0.69$, $p<0.001$)

6.3.7 Global DNA methylation

The median percentage 5-mc in leukocyte samples from study time point 1 was 3.34, and 2.00 at time point 2. The natural logarithms of the values were taken to improve model residuals, and outliers greater than 2 standard deviations from the mean were excluded, resulting in data from 38 dogs (34 male, 4 female) at time point 1 and from 24 dogs (23 male, 1 female) at time point 2 remaining for analyses.

Percentage 5-mc at time point 1 (5mc1) was significantly correlated with percentage 5-mc at time point 2 (5mc2) (Spearman's $\rho=0.52$, $p=0.01$, Figure 6.14). In addition, the change between 5mc1 and 5mc2 was significantly correlated with 5mc1 (Spearman's $\rho=0.50$, $p=0.02$), such that those with higher 5mc1 had the greatest percentage loss between the two time points. Correction for regression to the mean was performed on the change, using the same equation as in chapter 5. The relationship between 5mc1 and the change in 5mc between the two time points remained significant (Spearman's $\rho=0.51$, $p=0.01$).

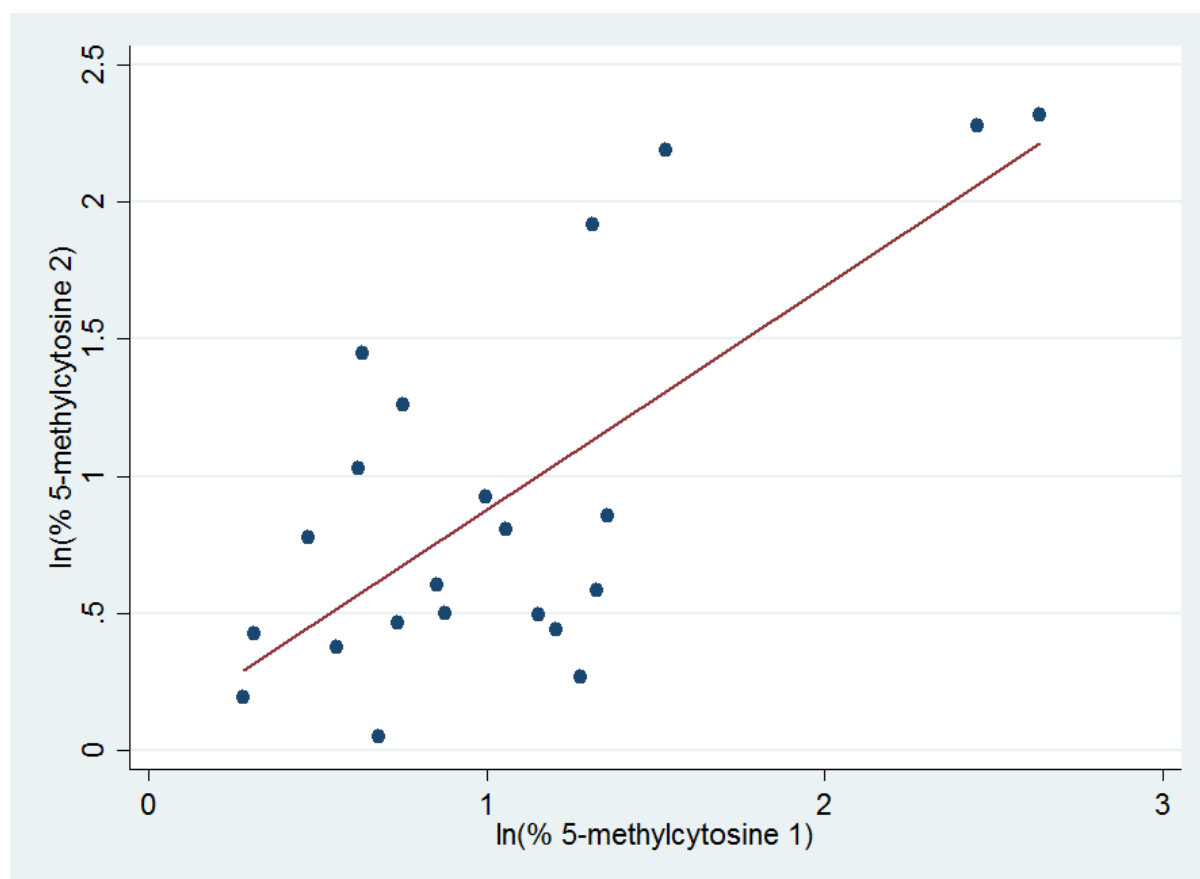


Figure 6.14 % 5mc at time points 1 and 2

There was a significant correlation observed (Spearman's $\rho=0.52$, $p=0.01$. $N=24$, 23 male, 1 female)

Age significantly increased with leukocyte percentage DNA methylation at time point 1 (5mc1) ($AIC=72.35$, $p=0.03$, Figure 6.15). LE adjusted age also increased with 5mc1, but the linear model was not significant ($AIC=56.86$, $p=0.21$, Figure 6.16). In addition, at time point 2, the linear model of age to 5mc2 was statistically significant ($AIC=53.31$, $p<0.001$), but the model including LE adjusted age was not significant ($AIC=38.95$, $p=0.50$). The change in 5mc between the two time points was not related to age ($F<0.001_{(1,20)}$, $R^2<0.001$, $p=0.98$) or LE adjusted age ($F=0.71_{(1,13)}$, $R^2=0.05$, $p=0.41$).

5mc1 significantly increased with both HNC1 ($AIC=65.74$, $p=0.002$, Figure 6.17) and HCC1 ($AIC=62.58$, $p<0.001$, Figure 6.18). Both models were improved by including

age (HNC1: AIC=63.47, $p<0.001$; HCC1: AIC=61.64, $p<0.001$). However, at time point 2 neither HNC2 or HCC2 were significantly modelled to 5mc2 (HNC2: AIC=54.64, $p=0.63$; HCC2: AIC=54.50, $p=0.55$). The change between 5mc1 and 5mc2 was unrelated to HNC2 ($F=0.20_{(1,20)}$, $R^2=0.01$, $p=0.66$) or HCC2 ($F=0.06_{(1,20)}$, $R^2=0.003$, $p=0.81$).

Briefly, analyses of potential further explanatory variables were undertaken. Weight was unrelated to 5mc1 (AIC=76.55, $p=0.41$, Figure 6.19), or the change between 5mc1 and 5mc2 ($F=0.08_{(1,20)}$, $R^2=0.004$, $p=0.78$), but was significantly positively related to 5mc2 (AIC=54.29, $p<0.001$). Percentage weight change between the two time points was not significantly related to 5mc2 (AIC=54.82, $p=0.79$) or the change between 5mc1 and 5mc2 ($F=0.01_{(1,20)}$, $R^2<0.001$, $p=0.92$). Cephalic index was significantly positively related to 5mc1 (AIC=46.45, $p<0.001$, Figure 6.20), and 5mc2 (AIC=37.90, $p<0.001$). In addition, cephalic index was significantly negatively related to 5mc1 minus 5mc2 ($F=5.99_{(1,13)}$, $R^2=0.32$, $p=0.03$, Figure 6.21), with dogs with lower cephalic indices reducing in 5mc percentage between the two time points, but those with higher cephalic indices tending to have an increase in 5mc percentage. Age was no longer significantly related to 5mc1 when cephalic index was included as an explanatory variable. Also, HNC2 and HCC2 were not significantly modelled to 5mc2 when cephalic index was included. HNC1 was no longer significantly related to 5mc1 when cephalic index was included, however HCC1 was still significantly associated with 5mc1 (AIC=42.70, $p<0.001$). Pure breed dogs had significantly higher 5mc1 than cross bred dogs (Wilcoxon rank sum test, $p<0.001$). However, pure breed dogs tended to have a higher cephalic index.

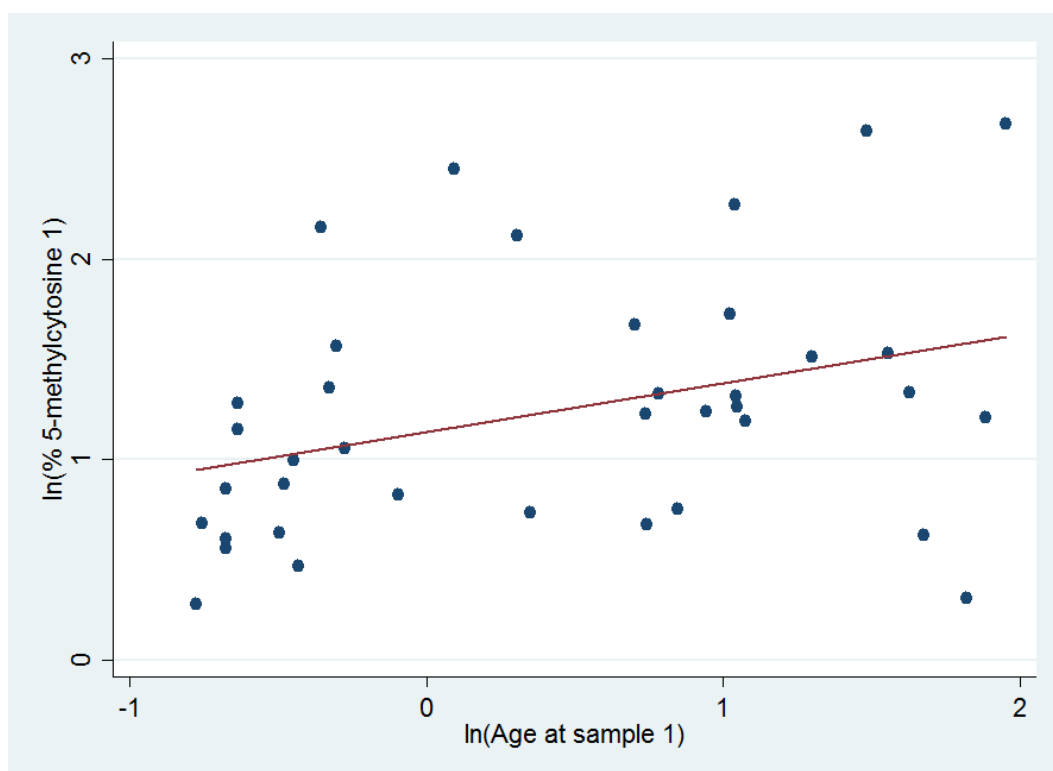


Figure 6.15 %5mc1 by dog age

%5mc at time point 1 was positively related to age of dog (AIC=72.35, $p=0.03$). N=38 (34M, 4F)

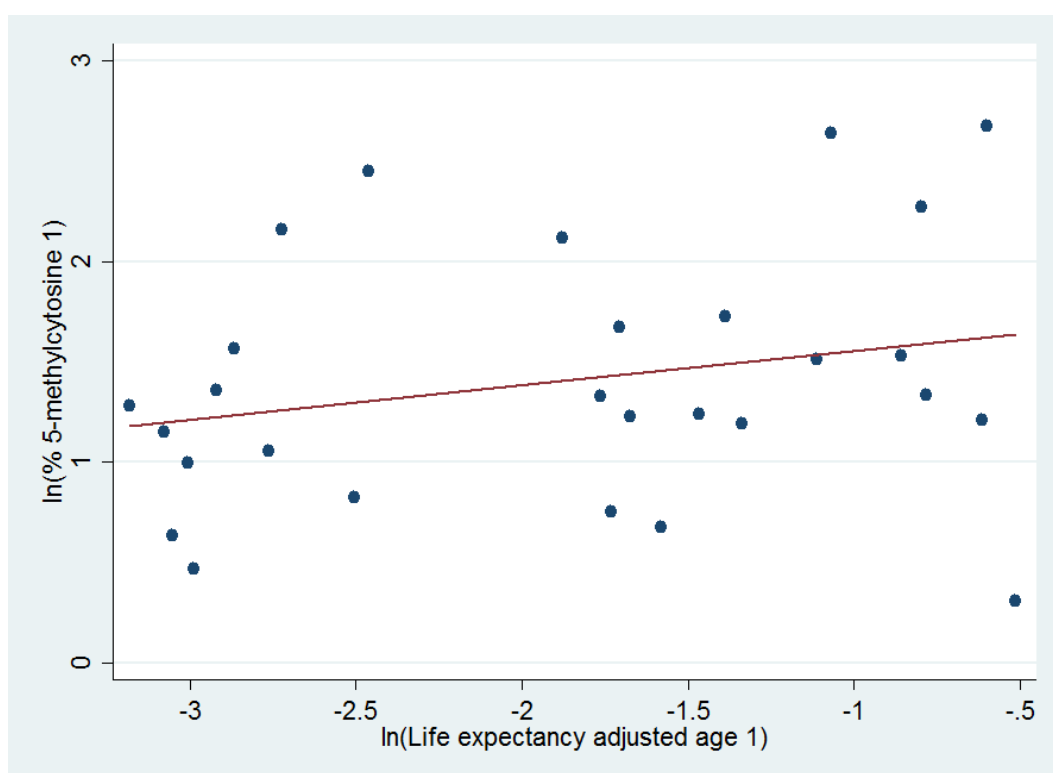


Figure 6.16 %5mc1 by dog life expectancy adjusted age

%5mc at time point 1 was not significantly associated with life expectancy adjusted age (AIC=56.86, $p=0.21$). N=28 (24M, 4F).

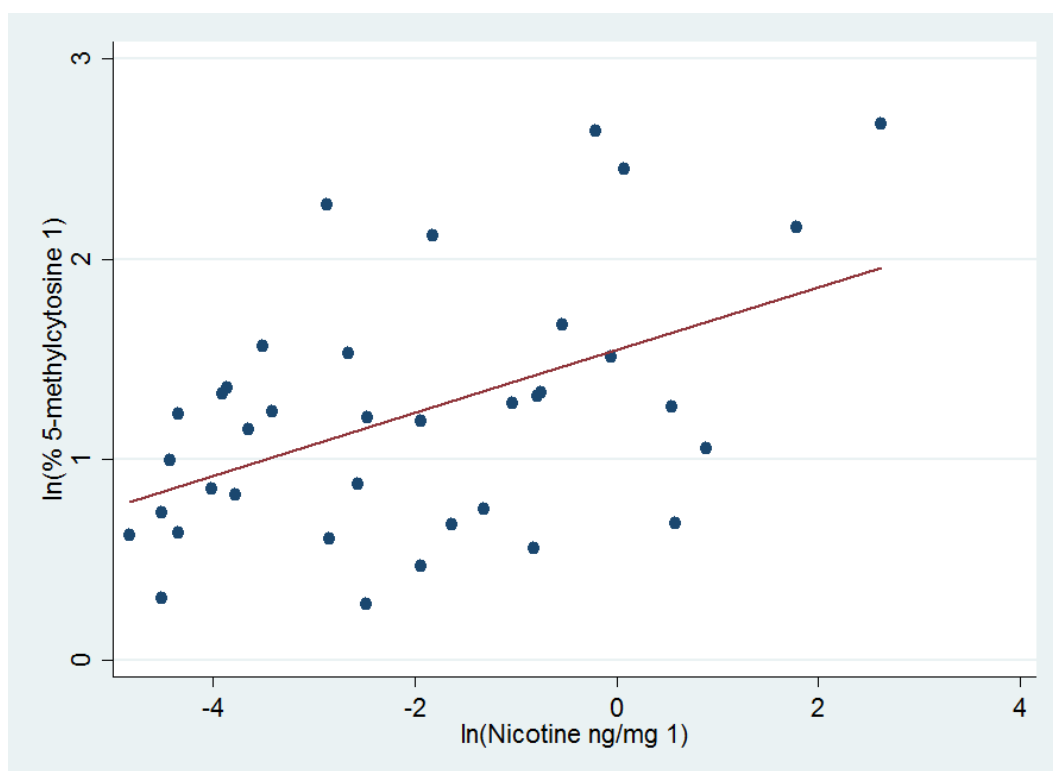


Figure 6.17 %5mc1 by hair nicotine concentration

%5mc at time point 1 was significantly positively associated with hair nicotine concentration (AIC=65.74, $p=0.002$). N=37 (34M, 3F)

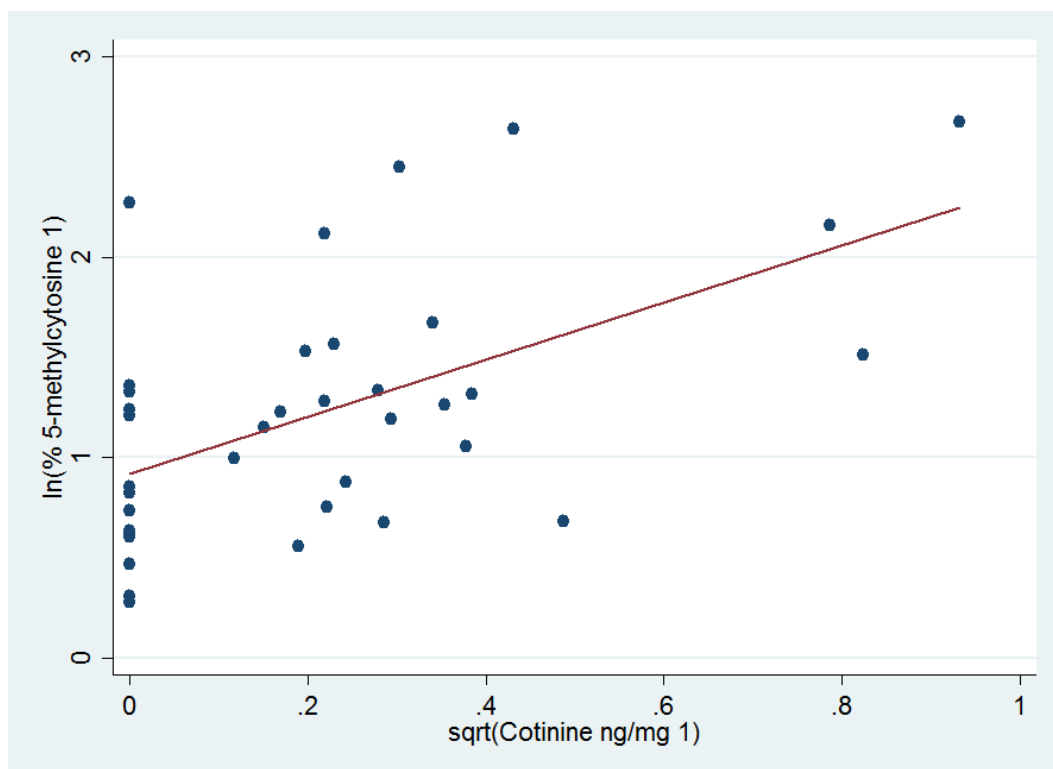


Figure 6.18 %5mc1 by hair cotinine concentration

%5mc at time point 1 was significantly positively associated with hair cotinine concentration (AIC=62.58, $p<0.001$). N=37 (34M, 3F)

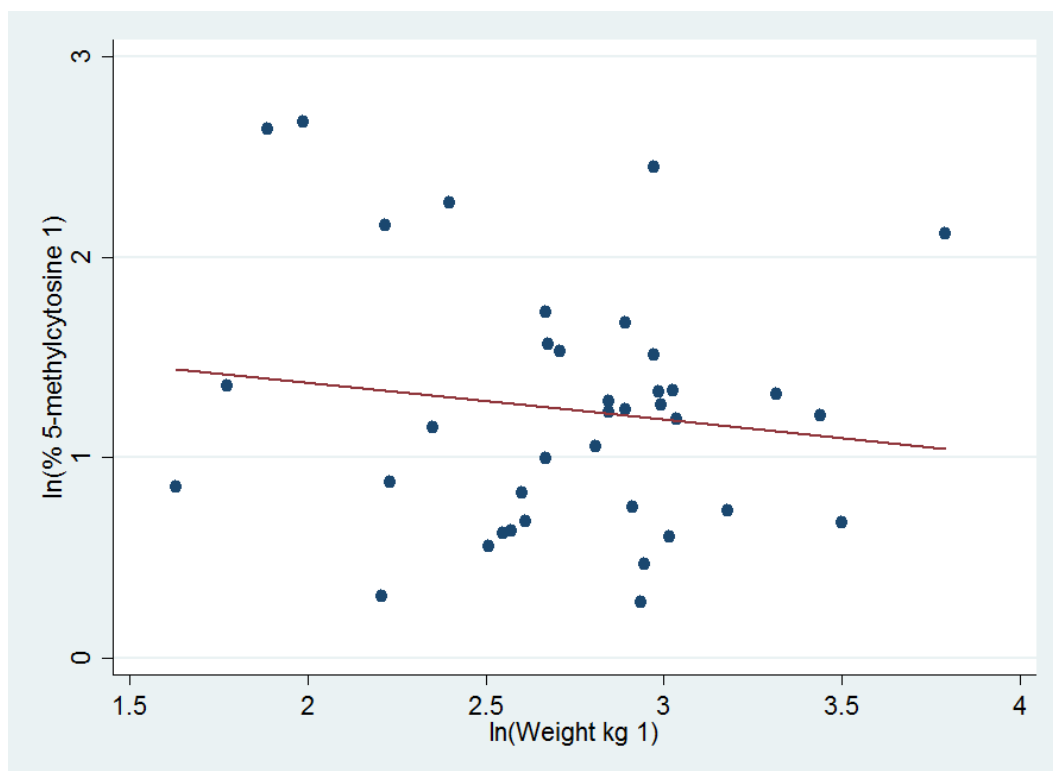


Figure 6.19 %5mc1 by dog weight

%5mc at time point 1 was not significantly related to dog weight (AIC, $p=0.34$). N=38 (34M, 4F)

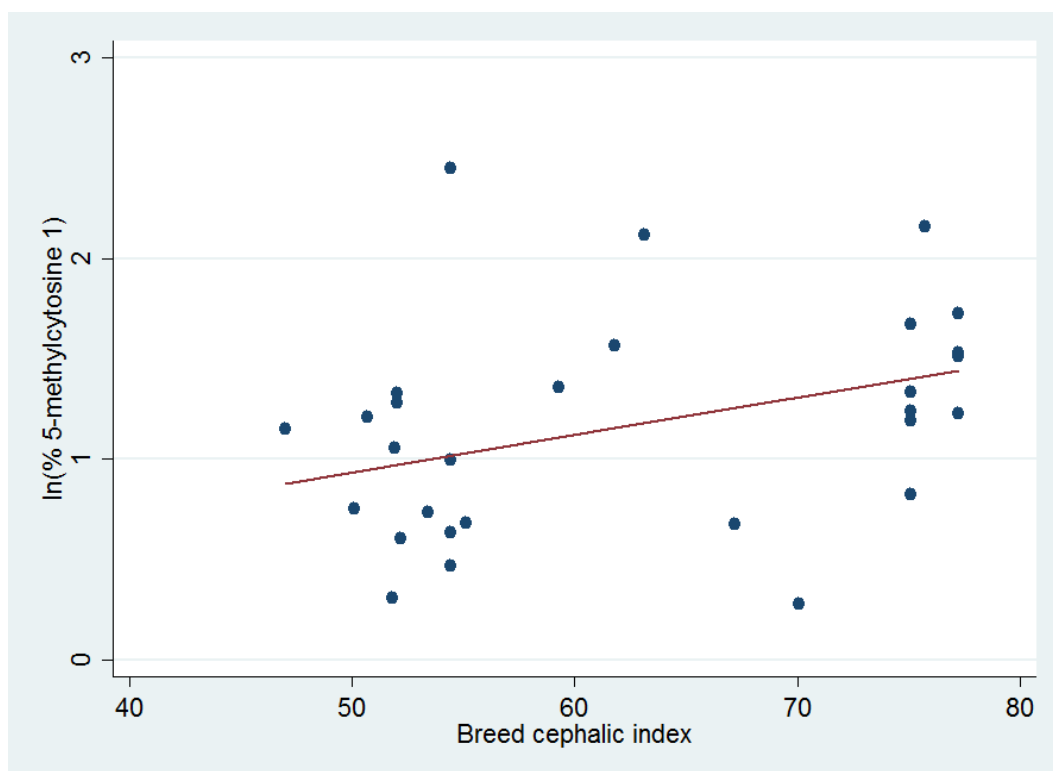


Figure 6.20 %5mc1 by breed cephalic index

%5mc at time point 1 was positively associated with cephalic index (AIC=46.45, $p<0.001$). N=29 (25M, 4F)

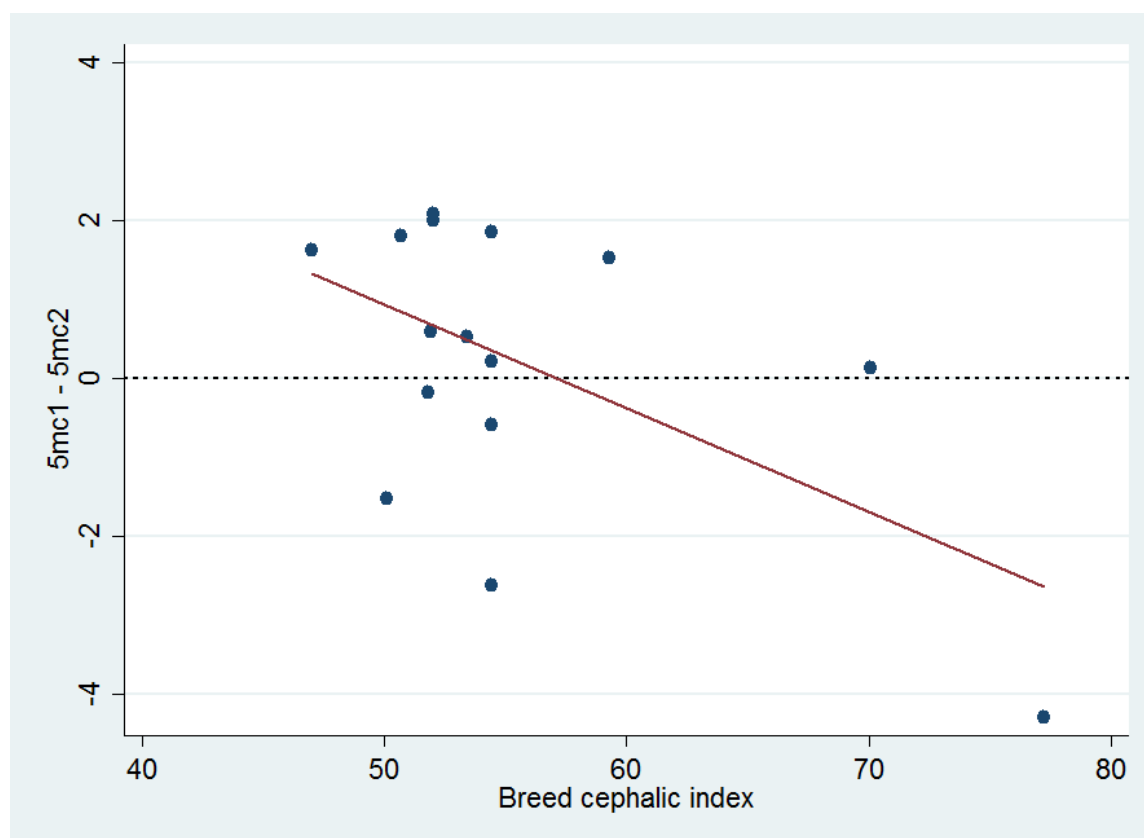


Figure 6.21 The difference between %5mc1 and %5mc2 by breed cephalic index

Cephalic index was significantly associated with the change in %5mc ($N=15$, $R^2=0.32$, $p=0.03$)

6.3.8 Relationships between mRNA expression levels, global DNA methylation and telomere length

Telomere lengths measured in chapter 5 were compared to gene expressions and 5-mc measurements in this chapter. Table 6-4 shows the correlation coefficients from correlation testing between mRNA expression of each gene and either telomere length or 5mc1. 5mc1 was significantly correlated with 3BHSD mRNA expression (Spearman's $\rho=0.48$, $p=0.004$) and significantly negatively related to TERT mRNA expression (Spearman's $\rho=-0.50$, $p=0.004$). In addition, a linear model of 5mc1 to TERT expression was highly significant when cephalic index was included as an explanatory factor ($F=184.5_{(3,20)}$, $R^2=0.97$, $p<0.001$). CDKN2A, 3BHSD, 17BHSD, sirtuin 1 and StAR mRNA expression were not significantly correlated to any of the telomere length measurements. Cremaster muscle RTL was significantly negatively correlated with AHR mRNA expression (Pearson's

correlation coefficient= -0.42 , $p=0.01$). AHR expression was negatively related to dog weight and cremaster muscle was positively related to weight, which was considered as a possible explanation. However, in a linear model of AHR to cremaster muscle, when weight was included as an explanatory variable the negative relationship between AHR and cremaster muscle remained significant. Similarly, AHR expression was negatively related to breed life expectancy and cremaster muscle telomere length was positively related to breed life expectancy. When life expectancy was included as an explanatory variable, the relationship between AHR and cremaster muscle telomere length was no longer significant. Clusterin was significantly correlated with buccal telomere length at time point 1 (Spearman's $\rho=0.38$, $p=0.03$). This relationship remained significant when several other explanatory factors were included. P450 SCC mRNA expression was significantly correlated with cremaster muscle telomere length (Pearson's correlation coefficient= 0.42 , $p=0.01$). This relationship also remained significant on further testing. TERT mRNA expression was significantly negatively correlated to vas deferens telomere length (Pearson's correlation coefficient= -0.49 , $p=0.004$). This relationship remained significant when further explanatory variables were included in linear models between the two, and, in addition, both HNC and TERT were significant explanatory variables in a linear model to vas deferens telomere length ($F=9.39_{(2,29)}$, $R^2=0.39$, $p<0.001$).

5mc1 was not significantly correlated to LRTL1 ($p=0.49$), BRTL1 ($p=0.53$) or CMRTL ($p=0.67$). Correlation between 5mc1 and ERTL was close to being statistically significant (Spearman's $\rho=-0.34$, $p=0.06$). 5mc1 and VDRTL were significantly positively correlated (Spearman's $\rho=0.39$, $p=0.03$). In addition, a linear model of 5mc1 to VDRTL was significantly improved by including HNC as an explanatory variable ($F=9.89_{(2,29)}$, $R^2=0.41$, $p<0.001$). 5mc2 was not significantly correlated to LRTL2 (Spearman's $\rho=0.38$, $p=0.09$) or BRTL2 (Spearman's $\rho=0.13$, $p=0.61$).

Table 6-4 Correlations between gene expressions and telomere length and %5mc1**Correlation coefficients are displayed, with significance level *=p<0.05, **=p<0.01**

	CDKN2A	3 β HSD	17 β HSD	AHR	Clusterin	p450 SCC	Sirtuin 1	StAR	TERT
Leukocyte RTL1	-0.15	0.005	-0.15	-0.02	0.3	0.08	-0.05	-0.1	-0.01
Cremaster muscle RTL	-0.09	0.16	0.04	-0.42*	0.29	0.42*	0.1	0.24	0.13
Buccal RTL1	-0.05	-0.12	-0.11	-0.18	0.38*	0.008	-0.17	-0.01	-0.08
Epididymis RTL	-0.14	-0.24	-0.11	-0.18	0.25	0.004	0.03	0.26	0.08
Vas deferens RTL	0.22	0.03	0.07	-0.32	-0.03	-0.19	-0.21	0.04	-0.49*
% 5-mc 1	0.14	0.48**	0.05	-0.03	-0.28	-0.25	-0.27	-0.11	-0.50**

6.4 Discussion

6.4.1 CDKN2A and ETS exposure

An increase in CDKN2A expression with ETS exposure was observed, in keeping with results previously seen in humans with pack years of smoking (Liu *et al.*, 2009). Smoking has been linked to cellular senescence (Tsuji, Aoshiba and Nagai, 2004; Nyunoya *et al.*, 2006), so it logically follows that CDKN2A levels would increase in exposed dogs. No significant change in CDKN2A expression was seen with ageing in testis, which was also seen when CDKN2A levels in mouse testes in 2.5 month old and 26 month old C57BL/6 mice (Krishnamurthy *et al.*, 2004). In that study, they did observe significant age related changes in other organs such as kidney and ovary. It may be that this marker would be a viable ageing biomarker in dogs if RNA from another tissue was used or if female reproductive tissues were studied. CDKN2A expression levels decreased with life expectancy and weight on initial analysis, but these variables were no longer significant when if the dog was a Staffordshire bull terrier or not was corrected for. ETS exposure still impacted expression regardless of breed, though. Index of deprivation was significantly related to CDKN2A, but not when HCC was included. However, in the absence of ETS data in future studies, having a postcode to generate an index of deprivation may be a helpful guide of exposure.

6.4.2 Clusterin expression and ETS exposure

Clusterin mRNA decreased with HCC, which was unexpected given that only one previous study demonstrated a decrease in clusterin with smoking (Antonelou *et al.*, 2011). In other studies, either there was no association demonstrated, or an increase in clusterin was observed with smoking (Carnevali *et al.*, 2006; Doyle *et al.*, 2010). It was unexpected that the expression of clusterin decreased with ETS exposure, as this marker has been observed to increase with senescence or in the presence of stress-inducing agents (Viard *et al.*, 1999; Petropoulou *et al.*, 2001). In addition, clusterin expression decreased with life expectancy adjusted age. It was anticipated that clusterin would increase with age, due to its link with

senescence, however, many of the dogs were pubescent so this may explain the decrease. An increase may have been observed if the dogs were older. In addition, a model of clusterin to 3BHSD was highly significant, which may be in keeping with this idea, that the levels are being impacted on by puberty. Clusterin mRNA levels also increased with dog weight. When clusterin protein levels were measured in plasma of 37 obese dogs, unlike in this study there was no association with body weight, but levels were seen to decrease with weight loss (García-Martínez *et al.*, 2012). Another aspect which may have caused a disconnect between the hypothesis and results, was that clusterin studies related to age in the literature that could be found are rodent studies so findings in other studies may not extend to dogs.

6.4.3 AHR expression and ETS exposure

AHR mRNA expression was hypothesised to increase with ETS exposure and decrease with age. While there was no significant relationship with unadjusted age, there was actually a significant positive relationship between AHR mRNA expression and life expectancy adjusted age. However, there was no significant relationship between AHR expression and ETS variables. It may have been worth measuring mRNA expression levels of genes downstream of AHR. For example, Fowler *et al.* (2014) did not find a significant difference in AHR mRNA expression level in foetuses exposed to maternal smoking, but did find a significant difference in COUP transcription factor 1, which has multiple AHR receptor sites. Staffordshire bull terriers had significantly higher AHR mRNA expression levels. Variables such as weight were no longer significantly related to AHR expression when Staffordshire bull terrier or not as a variable was included, so the difference seen in Staffordshire bull terriers is unlikely due to confounding factors. By having increased AHR expression, it is not known whether this breed would be at higher risk of developing malignancies through exposure to ETS. Shimizu *et al.* (2000) found that when AHR-deficient transgenic mice and AHR-positive mice were injected with benzo[a]pyrene (a tobacco smoke constituent), or had benzo[a]pyrene applied topically, AHR-deficient mice did not develop tumours, whereas those with AHR present developed subcutaneous tumours from the

injection and skin tumours from the topical application. In addition, when transgenic mice with constitutively active AHR and wild type mice were treated with an injection of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, an AHR ligand and hepatotoxin, there was only one small liver tumour found in 15 wild type mice and 19 tumours found in 18 transgenic mice (Moennikes *et al.*, 2004). However, Fan *et al.* (2010) found that, in the absence of xenobiotic ligands, AHR served as a tumour suppressor. This is in keeping with its link with cell cycle arrest and inhibition of cell proliferation. Transgenic AHR deficient mice were seen to significantly develop more liver tumours than wild type mice, when treated with a liver carcinogen which is not an AHR ligand. So, while having increased AHR expression may lead to a greater risk of tumours on exposure to AHR ligands, there may be a protective effect against tumours due to other causes.

6.4.4 Sirtuin 1 expression and ETS exposure

Sirtuin 1 mRNA expression was hypothesised to decrease with age and ETS exposure. The study dogs demonstrated an initial increase in expression with age up to around 1 year-old, then a decrease. This may have been linked with an increase with puberty, followed by a gradual decrease post-puberty. Once age was corrected for, there was a slight decrease in sirtuin 1 with ETS exposure. A gradual decrease could be observed with HNC1 and HCC1 when dogs a year old and over were included only, but this relationship did not reach significance, perhaps due to the small numbers above age 1. In terms of future studies using testes, the pattern with age would be an issue given that many owners opt to castrate their dogs when unwanted sexual behaviours start to occur. Sirtuin 1 expression was also seen to be positively associated with weight. No studies could be found on dog size and sirtuin 1 expression, however in cats, which share 95.3% similarity in sirtuin 1 sequence, an increase in body weight was positively associated with leukocyte sirtuin 1 mRNA expression (Ishikawa *et al.*, 2013). In addition, sirtuin 1 is linked to metabolism and sirtuin 1 knockout mice were demonstrated to be smaller than their wild type counterparts (Boily *et al.*, 2008). Basal metabolic rate differs between dog breeds (Speakman, van Acker and Harper, 2003), and sirtuin 1 levels may increase with dog breed size to meet the dog's metabolic needs. Life

expectancy was no longer related with sirtuin 1 when weight was included in the analysis.

6.4.5 TERT expression and ETS exposure

The linear models between unadjusted age or life expectancy adjusted age and TERT expression were not statistically significant. HNC1 was also unrelated to TERT. Sirtuin 1 and TERT expressions were not significantly correlated as may have been expected, but a linear model of TERT and dog weight to sirtuin 1 was significant ($p < 0.001$). Sirtuin 1 maintenance effects on telomeres have been shown to be mainly due to telomerase activity (Palacios *et al.*, 2010) and it may be interesting to measure telomere length in the testis itself and to see if sirtuin 1 and TERT expressions interact with telomere length in this tissue.

6.4.6 Steroidogenic enzymes expressions and ETS exposure

StAR and 17BHSD expression levels decreased with age, although not significantly. This may be in part due to the small numbers in the study, and in terms of life expectancy adjusted age, the smaller numbers for which breed life expectancy was known. Life expectancy adjusted age came close to being significantly modelled to StAR expression, with a p value of 0.08, and a pattern of descent could be observed when plotted. StAR and 17BHSD also decreased non-significantly with HNC and HCC. It would be interesting in future studies to see these relationships with increased study numbers, to see if the pattern of decrease in expression with age and ETS exposure would be replicated. 3BHSD was expected to decrease with both age and ETS exposure, however both these factors caused an increase in expression (HNC and HCC non-significantly). This could be due to expression increasing with puberty. Possibly, the expression levels may start to decrease at an older age, however the plot of expression against life expectancy adjusted age does not seem to plateau towards the older ages.

17βHSD expression was significantly higher in Staffordshire bull terriers. The other enzymes were not significantly different in Staffordshire bull terriers, so without further experiments it is not known if this would correspond to increased testosterone production in this breed. Therefore, this relationship was explored in Chapter 7. In addition, the levels of CDKN2A mRNA were lower in this breed, indicating lower levels of cellular senescence. Correlation testing between 17βHSD and CDKN2A mRNA expression was not statistically significant, but a linear model of 17βHSD to CDKN2A expression including whether the dog was a Staffordshire bull terrier or not as an explanatory variable was statistically significant ($F=6.14_{(3,32)}$, $R^2=0.37$, $p=0.002$).

The other breed relationship which was seen was that pure breed dogs had reduced TERT and clusterin mRNA expression levels, compared to cross breeds. It is difficult to reason why this would be, given that this comparison existed between a range of breeds and crosses. One possibility is that it is immune related; cross breeds generally have a greater mixture of canine major histocompatibility complex alleles than pure breed dogs (Kennedy *et al.*, 2002). MHC defects have been shown to have a link with spermatogenesis in human studies (van der Ven *et al.*, 2000), so may be related to different gene expression levels in the dogs.

6.4.7 mRNA expression level measurement methodology

The qRT-PCR measurement calculations are based on the reference gene having a stable expression level. However, studies have shown that expression of some commonly used reference genes are not constant throughout different tissues. For example, GAPDH, which was used here, was found to vary up to 15 times in expression between a panel of 72 human tissues (Barber *et al.*, 2005). Therefore, expression levels of various reference genes in different species and tissues have been measured in an effort to find which genes are optimum to use for the samples in question. Unfortunately, no studies could be found on stability of reference genes in canine testis. It may have been wise to establish which

reference genes had the most stable expression prior to measuring the relative expressions of genes in the study samples, to improve precision and avoid error due to variation of the reference gene expression. Software such as geNorm and Normfinder have been used for this purpose in previous canine studies (e.g. Schlotter *et al.* 2009; Wood *et al.* 2008). In addition, when levels of reference genes were studied in murine testes, β -actin mRNA levels varied significantly during development (O'Shaughnessy, Willerton and Baker, 2002). If there had been any effect of puberty and growth on the reference genes in the study dogs, this may have masked age related changes in the target genes. On a practical level, to establish the best reference gene combination, testis RNA would have needed to be collected from an age range of dogs and from different breeds which would be time consuming to collect. A range of breeds would be best to check due to the breed effect found on the telomere assay in chapter 4 and 5, with miniature schnauzers having unusual results. An age range would need to be examined to check for growth and puberty related changes. One positive aspect of the assay design was that the target genes were measured relative to two reference genes. It has been said that the use of multiple reference genes are required for accurate expression levels to be measured (Vandesompele *et al.*, 2002).

6.4.8 Global DNA methylation

Percentage 5-mc initially appeared to increase with age, although age was no longer significantly related when cephalic index, which happened to generally increase with age, was included in the analysis. In the existing study which could be found examining DNA methylation in dogs, 5-mc increased with age (Gryzinska *et al.*, 2016). In humans, there is a general loss of methylation with age, but it has been demonstrated that this is not the whole story. Loci in CpG islands gain methylation with age, whereas loci not in CpG islands lose methylation with age (Christensen *et al.*, 2009). The CpG content in dogs is increased relative to humans (Han and Zhao, 2009), which may explain why 5-mc did not decrease with age in this study and in Gryzinska *et al.* (2016). There was a relationship observed between cephalic index and 5-mc. The information for cephalic index in the dogs was incomplete, with previously published data used to obtain the index figures.

In addition, no dogs with very high cephalic index, such as a French bulldog, or a very low cephalic index, such as a greyhound, were included in the study. A potential future study could involve measurement of 5-mc level in dogs of a wide range of cephalic indices, measuring the index clinically to obtain a more accurate measure. There is also no obvious reason why cephalic index would be more linked to 5-mc than age, or any published material which may suggest a cause. No relationship with weight was found, which was also the case in Grynska *et al.* (2016). Percentage 5-mc increased with ETS markers, with HCC remaining significant when cephalic index was included in the analysis. Global 5-mc percentage was hypothesised to decrease with ETS exposure, however this may be due to the CpG island content in dogs again. A way to investigate this further, without using an expensive whole genome sequencing technique, would be to measure the methylation status of one gene of interest. Methylation of aryl hydrocarbon receptor repressor has been shown to be strongly associated with smoking (Monick *et al.*, 2012; Gao *et al.*, 2016). Changes in this gene would be interesting to compare with markers of ETS exposure.

An ELISA method to measure global DNA methylation was employed in this study. The reasons for this were that this method is cheap, does not require specialist knowledge or equipment, it can be used in dogs and requires relatively small amounts of DNA compared to some other methods. Other whole genome methylation methods include mass spectrometry, high performance liquid chromatography-ultraviolet and pyrosequencing. These methods were not used due to cost and need for specialist equipment. The most popular method for methylation profiling uses array or bead hybridisation, and many commercial kits are available. However, these kits are not suitable for use with canine DNA. A possible future method to use in canine samples would be a methylation-specific PCR, which uses qPCR to calculate relative methylation. This method only provides information on a small amount of the genome, but is one that could be used in dogs and is not high in cost.

6.4.9 Chapter summary

This chapter has examined multiple potential biomarker of ageing candidates in dogs. Few published data in dogs in this area are currently available. By examining multiple markers, pilot data is now available to inform future studies, and to base power calculations on to calculate sample sizes needed.

Testis RNA was used for gene expression studies of 9 genes. This tissue would normally be discarded at a castration procedure and large quantities of RNA could be extracted, enabling multiple genes to be tested. However, issues in some of the genes were found in the changes around growth and puberty of the younger dogs.

Global DNA methylation was measured using leukocyte DNA. This marker was found to change with ETS exposure, and was easy and cost effective to measure. Further data would need to be gathered regarding the effects of breed cephalic index and gene specific changes to use this marker in the future, but it can be measured longitudinally so could be a potential marker to monitor dogs over time, given further investigation.

Chapter 7: Steroid hormone concentrations and weight change post neutering

7.1 Introduction

7.1.1 Testosterone and age

A decrease in blood testosterone concentration with age has been observed in multiple human studies. For example, Pirke and Doerr (1973) observed a steady decrease in plasma testosterone concentration with age in 84 human males aged 22 to 90 and the decline in sex steroid hormones with age was recently proposed as a biomarker of ageing in men (Walther *et al.*, 2016). Thus far, in studies of dog testosterone concentration, evidence of a decline with age seems to be lacking. A study of 15 beagle dogs, aged 3 to 14 years, found no significant relationship between age and serum testosterone concentration (Lowseth *et al.*, 1990). A further study using 96 dogs of various breeds aged 6 months to 11 years found no relationship between plasma testosterone concentration and dog age (Taha and Noakes, 1982).

7.1.2 Testosterone and smoking

In addition to age-related changes, in humans, relationships have been observed between testosterone concentrations and smoking habits, and in addition, in one dog study. Sofikitis *et al.* (1995) measured testosterone concentrations in blood samples obtained from the left testicular vein of 49 smokers, mean age 30.3, and 28 never smokers, mean age 33.1. They found a significantly lower testosterone concentration in the smoking group. In a study using blood samples from 3486 Danish men with a median age of 19, both exposure to tobacco smoke prenatally and their own smoking habits were found to be significantly associated with testosterone concentrations. If the men were exposed to ETS prenatally, but did not smoke themselves, there was an increase in free testosterone concentration observed compared to non-exposed prenatally. In blood, 98 to 99.5% of testosterone is protein bound, to sex hormone-binding globulin or albumin (Luetjens and Weinbauer, 2012). Free testosterone is calculated using a combination of testosterone and SHBG concentrations. If the men were exposed prenatally and they also smoked themselves, there was a decrease in free

testosterone seen, due to an increase in SHBG. If they were not exposed prenatally, but did smoke themselves, total testosterone concentration was higher than in non-smokers unexposed prenatally, but the free testosterone concentration was unchanged (Ravnborg *et al.*, 2011). Similarly, Field *et al.* (1994) found when they measured serum testosterone concentration of 1241 men aged 38 to 70, higher total testosterone concentration, but no change in free testosterone concentration, was found in smokers. In another study of 126 non-smoking and 178 smoking males with primary infertility, testosterone concentration was significantly lower with increased smoking habit (Mitra *et al.*, 2012). In the study of dog testosterone and the relationship to smoking, Mittler *et al.* (1983) fitted permanently tracheostomised male beagles with machines which held cigarettes and emulated a “standard puff profile” of smoking. Serum testosterone concentrations were seen to decrease with increasing degrees of tar and nicotine quantities in the cigarettes administered. 6 β -hydroxylase liver activity was stimulated by smoking, resulting in an increase in hepatic metabolism of testosterone.

7.1.3 Other factors related to testosterone concentration in dogs

Limited information is available on breed differences in testosterone concentration. Urhausen *et al.* (2009) measured testosterone concentration in a total of 27 dogs of 4 different breeds: beagle, fox terrier, Labrador retriever and Great Dane. Median ages for each breed ranged between 1.5 and 3.5. Testosterone concentration was higher in beagles than in fox terriers.

Seasonal variations in testosterone concentrations have been demonstrated previously. When the serum and seminal concentrations of 12 adult mixed-breed dogs aged 3 to 6 years were measured on a monthly basis for one year, seminal testosterone peaked in October and was at its lowest in April. In contrast, serum testosterone concentrations did not significantly change throughout the year (Albrizio *et al.*, 2013). In a similar study, plasma concentration of testosterone was measured in 4 mixed-breed dogs every 15 days for one year. The dogs were

housed individually in a vivarium under an artificial lighting schedule. A peak in testosterone concentration was observed in late August and early September, and concentrations were significantly higher at this point than other times of the year. The authors suggest this is related to the normal reproductive behaviour of the dog and other canids, due to the timings at which females go into heat (Falvo *et al.*, 1980). In contrast, when serum testosterone concentrations were measured in 7 dogs every 15 days over a 14-month time period in a study by Martins *et al.* (2006), a reduction in testosterone concentration was observed in the summer period. The study in question was conducted in a tropical zone, and the authors suggest that the summer weather of high temperature and high rainfall led to the reduced concentration in the summer. High temperatures would not have affected testosterone concentrations in our study, being based in Glasgow, but the author notes there is certainly high rainfall all year round. In addition to season, variation throughout the day has been observed. In 3 mature mixed-breed dogs housed individually in a vivarium with an artificial lighting schedule, blood samples were taken at 20 minute intervals, and were shown to vary from 0.4 to 6ng/ml over a 24-hour period. There was, however, no significant difference observed between samples taken in the light and dark periods (DePalatis, Moore and Falvo, 1978).

Another self-evident factor relating to testosterone concentrations in dogs is if they have been castrated or not. When DePalatis *et al.* (1978) measured plasma testosterone concentration in 2 castrated dogs, they found the concentrations to be below the lower limit of detection for their assay. Frank *et al.* (2003) measured testosterone concentrations in 20 intact and 30 castrated male dogs of various breeds. The median testosterone concentration was 2.8ng/ml in intact dogs, versus 0.04ng/ml in castrated dogs.

7.1.4 Cortisol and age

There are limited data on any association between cortisol concentration and age, and no studies examining the relationship between dog age and cortisol could be found. Salivary cortisol concentration was measured in 1671 adult humans aged

between 30 and 74 years. Cortisol was measured in samples taken in the morning and evening. Evening cortisol concentration was significantly higher in older subjects than younger subjects, and in older men than younger men in the morning (Larsson *et al.*, 2009). In addition, when hair cortisol concentration was measured in 654 adults aged 47 to 82, cortisol concentration significantly increased with age (Feller *et al.*, 2014). In two studies relating to cortisol concentrations in saliva of young children at home versus in childcare, there was no significant relationship between baseline cortisol concentration and age, only the change from baseline cortisol in response to stress, which will not be measured in this study (Dettling, Gunnar and Donzella, 1999; Watamura *et al.*, 2003).

7.1.5 Cortisol and smoking

In the study of hair cortisol concentrations of 654 adults by Feller *et al.* (2014) mentioned in the previous subsection, current smoking was associated with significantly higher cortisol concentration, even after age was included in the analysis. Badrick *et al.* (2007) examined salivary cortisol concentration in 3103 men and 1128 women. Current smokers had a higher release of total cortisol compared to never smokers. No significant relationship was observed between number of cigarettes smoked and cortisol concentration, but increased concentration of cortisol on awakening was seen in those with higher cigarette consumption. Granger *et al.* (2007) measured salivary cortisol concentrations in mothers and their infants. Cortisol concentrations were significantly higher in that of the mothers who smoked, but there was no significant difference in infant cortisol concentration based on the mothers' smoking habit. Varvarigou *et al.* (2009) measured cortisol concentration in cord blood of neonates of 100 smoking and 100 non-smoking mothers. Not only were the cortisol concentrations in cord blood in neonates with smoking mothers significantly higher, but there was also a positive linear association between cortisol and the number of cigarettes smoked by the mother per day.

7.1.6 Measuring cortisol in dogs

Cortisol concentration has been measured in a variety of dog tissues, including saliva, blood, faeces, and hair (Accorsi *et al.*, 2008; Bennett and Hayssen, 2010; Ouschan, Kuchar and Möstl, 2013). Due to the nature of the sample, dog hair cortisol concentration has been demonstrated to be representative of baseline cortisol concentrations over a period of time (Bryan *et al.*, 2013). Bennett & Hayssen (2010) measured cortisol concentrations in both hair and saliva of dogs. The concentrations in both tissues were significantly positively correlated. However, they also demonstrated differences in cortisol concentration in different colours of hair, with black dogs having lower cortisol concentrations in their hair than non-black dogs. This difference was not evident in the saliva of these dogs. Saliva cortisol concentrations were reported to be lower in neutered males and females than their intact counterparts in a study of 19 dogs. In addition, salivary cortisol concentrations were higher in small dogs than large or giant dogs (Sandri *et al.*, 2015).

7.1.7 Testosterone, cortisol and telomeres

Limited information could be found regarding the relationship between the hormones and telomere length. Bekaert *et al.* (2005) measured TRF length in 110 samples from an elderly healthy male human population (aged 71-86 years), and found no significant relationship between TRF length and free testosterone concentration. Needham *et al.* (2014) measured telomere length in 851 individuals and compared relationships with sex hormones to try to understand the observed gender difference in telomere length. No significant relationship could be found between testosterone and LTL in a multivariate model.

Choi *et al.* (2008) cultured peripheral blood mononuclear cells from 8 donors aged 25 to 55. They exposed the cells to increasing concentrations of hydrocortisone and found a significant negative relationship with telomerase activity levels. Zalli *et al.* (2014) measured LTL in 333 individuals aged 54-76, and divided the results

into two groups: long telomeres and short telomeres. There was no significant difference in saliva cortisol concentration between the groups. Saliva telomere length was measured in a group of 97 girls aged 10-14. There was no significant difference in salivary cortisol concentration between individuals with long and individuals with short telomeres, however individuals with short telomeres displayed a larger increase in cortisol concentration following a stressful stimulus (“cortisol reactivity”) (Gotlib *et al.*, 2015).

7.1.8 Dog weight gain after neutering

Neutered dogs are known to be at increased risk of obesity (Colliard *et al.*, 2006; Lund *et al.*, 2006). Studies have demonstrated a decrease in metabolic rate in neutered animals compared to intact animals (e.g. Root *et al.* 1996). Testosterone concentrations are associated with lean mass (Griggs *et al.*, 1989) and the reduction in concentration in males post-castration can lead to loss of lean mass. Various other factors have been suggested as risk factors for obesity, such as breed, household income, steps taken per day by the dog and owner age (Colliard *et al.* 2006; Kienzle *et al.* 1998; Lund *et al.* 2006; Warren *et al.* 2011). A factor which has not been discussed is owner smoking. Interestingly, recent studies have demonstrated a potential link between child obesity and parental smoking habits (e.g. Han *et al.* 2015; Robinson *et al.* 2016).

Given the link between obesity and oxidative stress (Vassalle *et al.*, 2009), a link between telomere length and obesity has been observed in multiple studies. When LTL was measured in 793 children aged 2 to 17 years of age, the mean LTL in obese children was 23.9% shorter than that of children classed as non-obese (Buxton *et al.*, 2011). Cui *et al.* (2013) measured LTL in 2912 Chinese women aged 40-70. Telomere length was found to be significantly inversely associated with weight, waist circumference, hip circumference, body mass index and waist to height ratio. In addition, telomere length was demonstrated to be shorter in adipose tissue cells with increasing body mass index (Moreno-Navarrete *et al.*, 2010). However, not all studies have found associations between obesity and telomere

length. For example, when LTL was measured in 1459 individuals with a mean age of 21.7 years, no association between telomere length and waist circumference or body mass index was found (Bethancourt *et al.*, 2017).

7.1.9 Chapter aims and hypotheses

Given the decrease in testosterone with age in humans, plasma testosterone concentrations were measured in male dogs at both time points to investigate the pattern with age in dogs. It was hypothesised that concentrations would be inversely related to ETS biomarkers and age. Aside from age, concentrations were anticipated to be lower at the second time point as this would be more or less a year after castration.

Psychological stress has been associated with early mortality in dogs (see Chapter 1). As a measure of chronic stress, cortisol hair concentration was measured at the second time point. A positive relationship was expected with ETS biomarkers, but variables such as hair colour also needed to be accounted for.

Potential links have been established between telomere length and obesity. The weight at both time points was also recorded, and the body condition score. Body condition scores were hypothesised to be increased in dogs living in smoking homes and to be negatively associated with telomere length.

7.2 Methods

7.2.1 Study dogs

Study dogs and demographic variables were as those described in chapter 5. Nicotine and cotinine concentrations were described in chapter 3. Male dogs were sorted into prepubescent, pubescent and postpubescent groups based on their age, size and physical examination by a veterinary surgeon.

7.2.2 Plasma testosterone concentration

Testosterone concentration was measured in spare plasma from blood samples taken as part of the health check at both time points, in the male dogs only. Concentrations were measured by a veterinary laboratory technician in the University of Glasgow Veterinary Diagnostic Service using a chemiluminescence method on an Immulite 2000 (Siemens, Erlangen Germany).

7.2.3 Measurement of cortisol concentration in hair

Hair clipped for blood sampling purposes at time point 2 was divided between that to be used for measurement for nicotine and cotinine concentrations, and to be used for cortisol concentration measurement. Hair for cortisol measurement was kept in sample envelopes at room temperature. Cortisol concentration was measured in hair samples from all dogs attending time point 2 appointments (24 males, 1 female), using an ELISA kit (R&D Systems, Minneapolis USA). Prior to performing the ELISA, the fur samples were prepared as following. A 20ml scintillation vial was half filled with dog hair sample. The hair was washed 5 times with water and twice with ethanol, for 30 seconds each wash and removing all liquid possible after each wash. The hair was then allowed to dry in a fume hood for 2 days. 35mg of hair was placed in a 2.5ml microcentrifuge tube. The hair was homogenised by adding two cone ball bearings, immersing the tube into liquid nitrogen for a minute then placing in a MM400 Ball Mill (Retsch, Düsseldorf Germany), milling the sample for 3 minutes at a frequency of 25Hz. This process

was repeated until the hair was powdered. 35-40mg of powdered hair was placed into a 2ml screw cap borosilicate glass vial, recording the weight of hair used. 1ml of HPLC-grade methanol was added to the powder and the vial sealed. The vial was placed in a shaking incubator at 52 °C, 125rpm for 16 hours. Following this, the powder was allowed to settle for 20 minutes and then 750µl of supernatant was transferred to a 15ml borosilicate test tube. The solution was then evaporated using a sample concentrator. 250µl of Calibrator Diluent (provided in ELISA kit) was added and the tube vortexed for 10 minutes. The ELISA was then performed as per the kit instructions. Reconstituted cortisol standard (kit provided) was used to make 7 concentrations of standard ranging from 10ng/ml to 0.156ng/ml for the standard curve. 100µl of the standards or sample was added to each well of a microplate (kit provided). Samples and standards were assayed in duplicate. In addition, non-specific binding wells were set up by adding 150µl of Calibrator Diluent to 2 wells and 100µl of Calibrator Diluent was added to a further 2 wells assigned as zero standard wells. 50µl of Cortisol Conjugate (kit provided) was then added to all wells, followed by 50µl of Primary Antibody Solution (kit provided) to all wells except for the non-specific binding wells. The plate was covered with an adhesive strip and incubated for 2 hours at room temperature on an orbital microplate shaker at 500rpm. The contents of the wells were removed and the wells washed 4 times using 400µl of 1X Wash Buffer (diluted to working concentration from kit provided buffer) each time. Substrate Solution was prepared by combining equal volumes of Color Reagents A and B (kit provided), protecting the solution from light. 200µl of Substrate Solution was added to each well and the plate incubated at room temperature for 30 minutes on the bench top, with the plate protected from light. 50µl of Stop Solution (kit provided) was added to each well. After 10 minutes, optical density was measured at 540nm using a microplate reader. The standard curve was generated using the optical density readings from the cortisol standards and used to find the corresponding cortisol concentrations for the test samples. The resulting concentration was corrected for dilution factors and adjusted for the input sample weight to obtain a final concentration of pg of cortisol per mg of hair.

7.2.4 Dog weight assessment

Dogs were weighed on an electronic scale at both time points. Percentage weight change was calculated, adjusting for expected weight gain due to growth. Body condition score (BCS) was assessed by a veterinary surgeon, and dogs with a BCS of 3.5/5 or greater were placed in the “overweight” group.

7.2.5 Data analyses

Linear models were analysed as previously described in chapter 4, using R (R Core Team, 2014). As testosterone at time point 1, cortisol concentration and weight gain data were non-parametric as determined by a Shapiro-Wilk test, group comparisons were tested with either Wilcoxon rank sum or Kruskal Wallis tests. Correlations involving these variables were examined using Spearman’s rank correlation coefficient testing.

7.3 Results

7.3.1 Plasma testosterone concentrations

As expected, pre-castration plasma testosterone concentrations (T1TC) were significantly higher than post-castration plasma testosterone concentrations a year later at time point 2 (T2TC) ($p < 0.001$, Wilcoxon rank-sum test). The median T1TC was 43nmol/L, compared to 1.68nmol/L for T2TC. The upper limit of detection of the assay was 56nmol/L, and 13 of the dogs had T1TC above this limit. Given the distribution of the data for T1TC, and it not being known how far above 56nmol/L the concentrations of T1TC in the 13 above upper limit dogs were, the T1TC was divided into 3 groups, summarised in Table 7-1.

Dogs with plasma testosterone concentration of above 56nmol/L were assigned a concentration of 56nmol/L for analyses and figures. There was a non-significant difference in age between the 3 groups (Kruskal-Wallis test, $p = 0.15$). As can be seen in Figure 7.1, there was a trend in increase of age across the groups. The dogs were sorted into pre-pubescent, pubescent and post-pubescent according to their size and age. Pre-pubescent dogs had the lowest median T1TC (13.4nmol/L), followed by pubescent dogs (30.2nmol/L) and post-pubescent dogs having the highest median T1TC (53.5nmol/L) (Figure 7.2). There was not a significant difference in T1TC between puberty statuses (Kruskal Wallis test, $p = 0.07$), but there was a significant difference in T1TC in pre-pubescent dogs compared to the other two puberty statuses combined (Wilcoxon rank-sum test, $p = 0.04$).

Table 7-1 Demographics of dogs grouped according to plasma testosterone concentration at time point 1

Median values are given, with inter-quartile range in brackets. Numbers of each breed and puberty status are listed. SS=Springer spaniel, GSD=German shepherd dog, BT=Border terrier, Ret/RW & BM X= Retriever/Rottweiler & bull mastiff X, SBT=Staffordshire bull terrier, PT=Patterdale terrier, LT=Lakeland terrier, CKCS=Cavalier King Charles spaniel, JRT=Jack Russell terrier, German SP=German shorthaired pointer, M.Schnauzer=miniature Schnauzer

	Group 1	Group 2	Group 3
T1 (nmol/L)	<20	20-56	>56
Age	0.45 (0.65)	1.36 (2.31)	2.02 (1.94)
Weight (kg)	14.4 (7.00)	17.2 (18.4)	18 (6.40)
HNC1	0.08 (0.78)	0.20 (0.41)	0.08 (0.56)
HCC1	0.01 (0.14)	0.05 (0.06)	0.00 (0.09)
Breeds	Basset hound (n=1) Beagle (n=1) Border collie (n=3) Collie/Labrador X (n=1) Pomeranian/BT X (n=1) Ret/RW & BM X (n=1) Shar-pei (n=1) SS/GSD X (n=1) SBT (n=1) Yorkshire terrier (n=2)	Bearded collie (n=1) Boxer (n=1) CKCS (n=1) Cocker spaniel (n=1) JRT (n=1) M. Schnauzer (n=1) PT/LT X (n=1) Rottweiler (n=1) Springer spaniel (n=1) SBT X Lurcher (n=1) SBT (n=1)	Border collie (n=1) BT/Pug X (n=1) CKCS (n=1) GSD/Collie X (n=1) German SP (n=1) PT (n=1) Springer spaniel (n=1) SBT X Lurcher (n=1) SBT (n=5)
Pre-pubescent	n=7	n=3	n=2
Pubescent	n=3	n=3	n=4
Post-pubescent	n=3	n=5	n=7

There was no significant difference between the T1TC groups in weight ($p=0.32$), hair nicotine concentration ($p=0.90$) or hair cotinine concentration ($p=0.70$). It was noted that there were 5 Staffordshire bull terrier dogs in group 3 (T1 of $>56\text{nmol/L}$). T1TCs were significantly higher in the Staffordshire bull terriers than the other dogs (Wilcoxon rank-sum test, $p=0.02$). If the Staffordshire bull terrier crosses were included with the pure Staffordshire bull terriers, there was again a significant difference with the other breeds of dog ($p=0.009$, Figure 7.3).

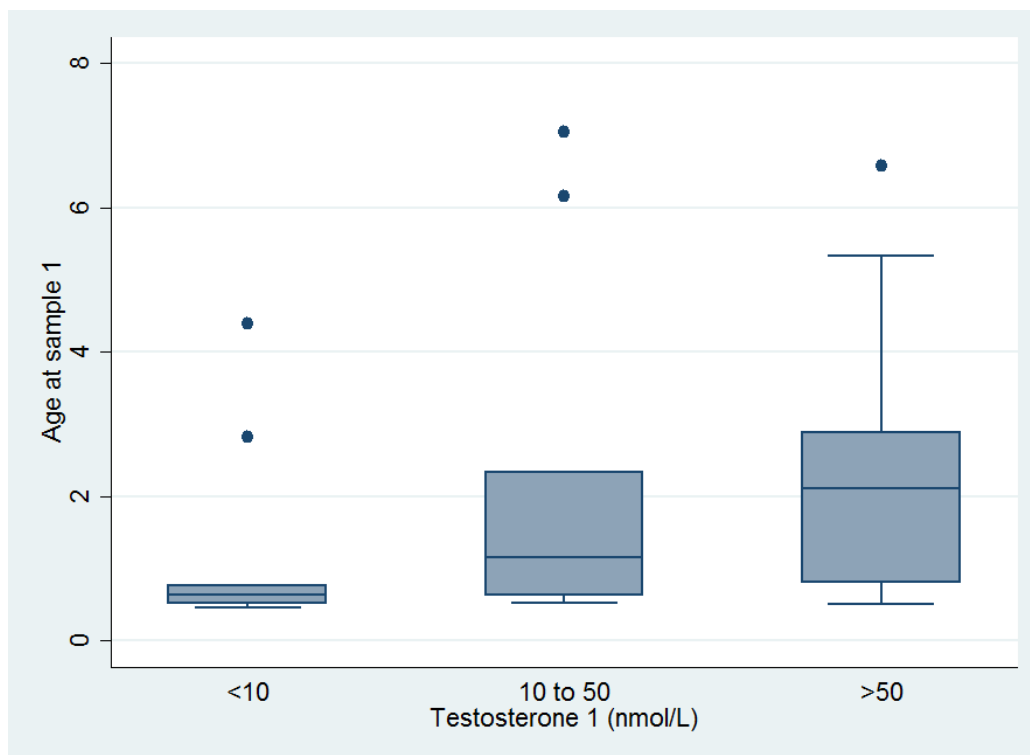


Figure 7.1 Box plot of dog ages in groups of plasma testosterone concentration (nmol/L) at time point 1.

The difference in age between groups of testosterone concentrations was not statistically significant ($p=0.15$). $N=37$.

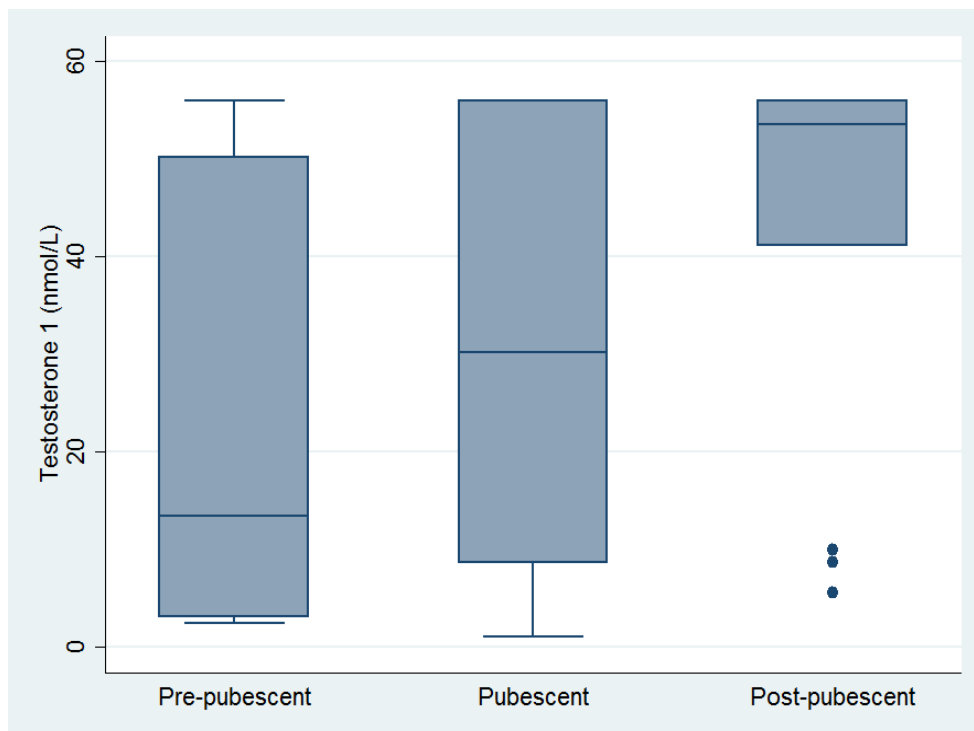


Figure 7.2 Box plot of plasma testosterone concentrations (nmol/L) at time point 1 in dogs by their puberty status.

There was no significant difference in testosterone concentration by puberty status ($p=0.07$). $N=37$.

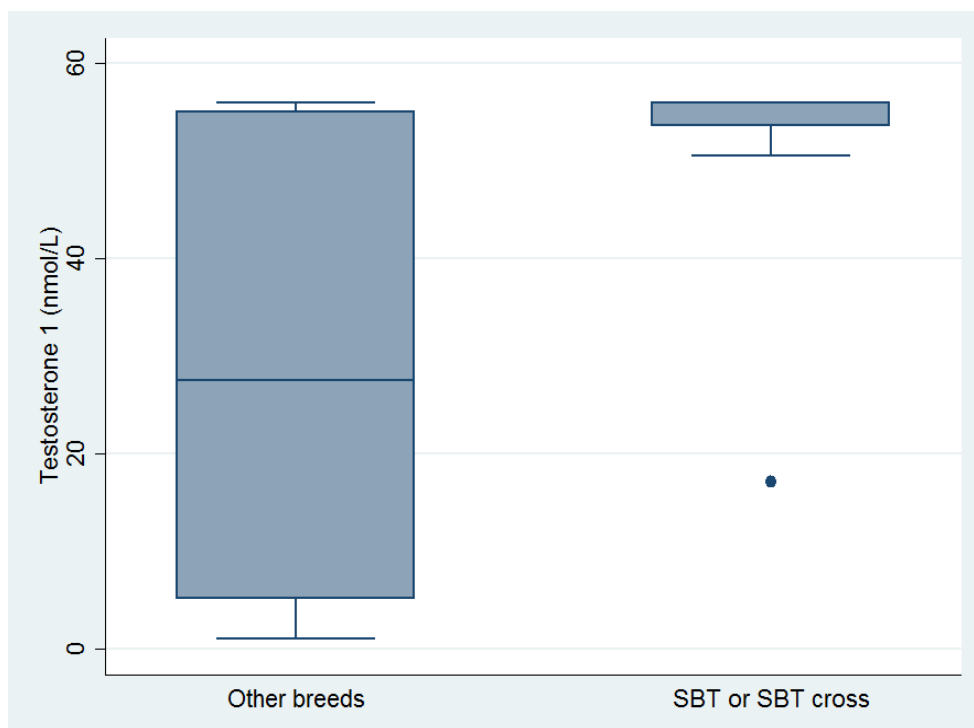


Figure 7.3 Box plot of plasma testosterone concentrations (nmol/L) at time point 1 by whether the dog was a Staffordshire bull terrier or cross

("SBT or SBT cross") or not ("Other breeds"). There was a significant difference in testosterone concentration between the two groups ($p=0.02$). $N=37$.

The correlation between T1TC and T2TC was not statistically significant (Spearman's $\rho = -0.17$, $p = 0.42$). Age was negatively related to T2TC ($F = 4.77_{(1,22)}$, $R^2 = 0.18$, $p = 0.04$, Figure 7.4). While dog weight was negatively related to T2TC, the relationship was not significant ($F = 2.57_{(1,22)}$, $R^2 = 0.10$, $p = 0.12$). However, weight gain in kilogrammes was negatively related to T2TC ($F = 5.67_{(1,22)}$, $R^2 = 0.20$, $p = 0.03$), as was percentage weight gain ($F = 4.48_{(1,22)}$, $R^2 = 0.17$, $p = 0.046$). The linear model of percentage weight gain to T2TC was significantly improved by including age ($F = 4.60_{(2,21)}$, $R^2 = 0.30$, $p = 0.02$). Too few Staffordshire bull terriers returned at time point 2 to test whether there was a significant relationship between the breed and T2TC. There was a significant positive relationship between T2TC and breed life expectancy ($F = 235.3_{(1,15)}$, $R^2 = 0.94$, $p < 0.001$). The linear models of hair nicotine concentration at time point 2 to T2TC ($F = 0.12_{(1,22)}$, $R^2 = 0.005$, $p = 0.74$), and cotinine concentration at time point 2 to T2TC ($F = 1.16_{(1,22)}$, $R^2 = 0.05$, $p = 0.29$) were not statistically significant.

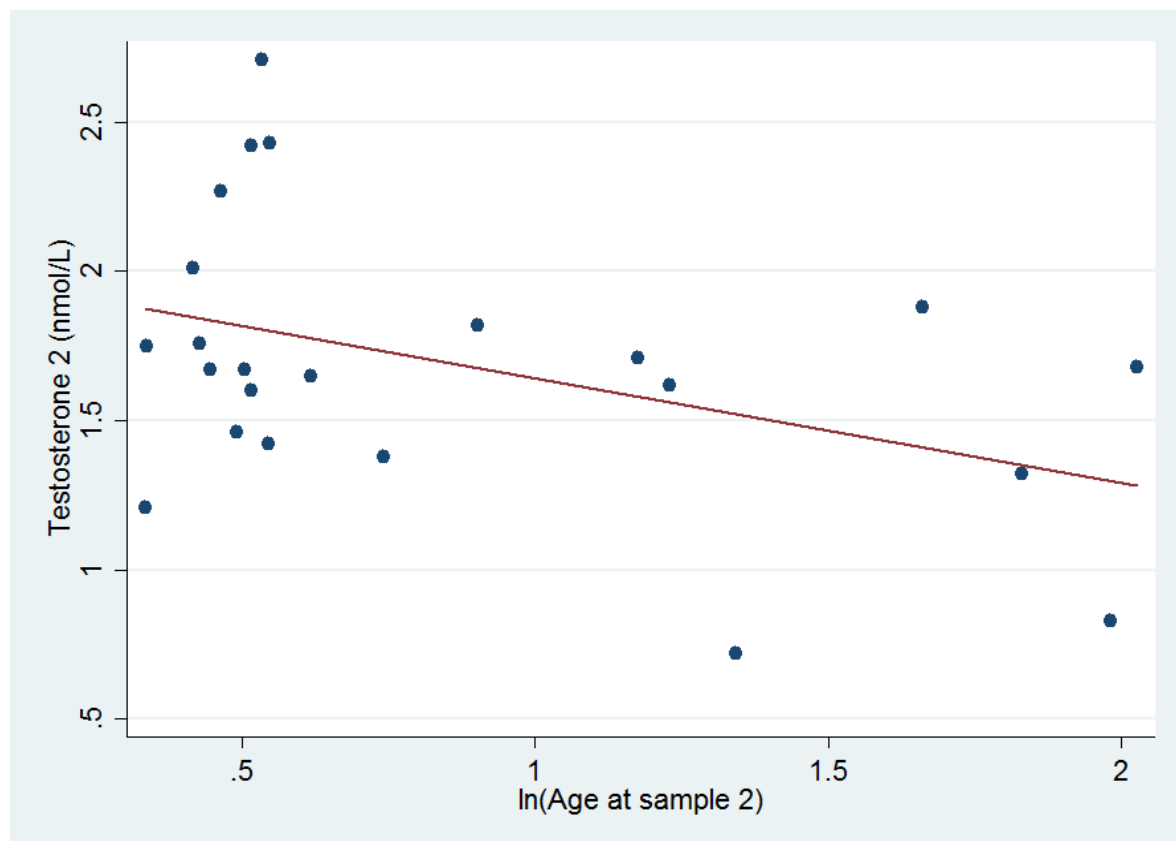


Figure 7.4 Plasma testosterone 2 by dog age

Scatter plot with regression line of plasma testosterone concentration (nmol/L) by natural log of dog age at time point 2. The linear model was statistically significant ($R^2 = 0.18$, $p = 0.04$). $N = 24$

Table 7-2 shows the beta coefficients of models of mRNA expression of genes measured in testis of the dogs to T1TC. Dogs with T1TC of over 56nmol/L were assigned T1TC of 56nmol/L for this analysis as there was no way of knowing how far above 56nmol/L their concentration of plasma testosterone was. 3BHSD and StAR, enzymes in the testosterone synthesis pathway, were significantly modelled to T1TC (3BHSD: AIC=327.01, $p<0.001$; StAR: AIC=323.98; $p<0.001$). In addition, mRNA expression levels of TERT and sirtuin 1 were significantly modelled to T1TC (TERT: AIC=321.27, $p<0.001$; sirtuin 1: AIC=324.74, $p<0.001$). Initially, p450 SCC mRNA expression was not related to T1TC (AIC=322.37, $p=0.72$). As p450 SCC catalyses conversion of cholesterol for the synthesis of hormones, cholesterol concentration was included as an explanatory variable in the model of p450 SCC mRNA expression to T1TC. This resulted in a significant model to T1TC (AIC=311.55, $p<0.001$). As AHR is known to be activated by chemicals in cigarette smoke, cotinine concentration was included as an explanatory variable in the model of AHR mRNA expression to T1TC. This model was statistically significant (AIC=316.45, $p=0.02$). In addition, as the genes were potential biomarkers of ageing, age was included in models of the gene expressions to T1TC to see if this impacted the relationships. When age was included, 17BHSD was significantly modelled to T1TC (AIC=324.57, $p=0.01$). This was also the case for the models of CDKN2A mRNA expression (AIC=324.07, $p=0.008$) and clusterin mRNA expression (AIC=323.73, $p=0.007$) to T1TC.

Table 7-2 Beta coefficients of linear models of the gene mRNA expressions to testosterone concentration at T1.

*** indicates significance level of $p < 0.001$.

Gene	β coefficient
3BHSD	18.61***
17BHSD	10.76
TERT	39.67***
StAR	33.89***
Sirtuin 1	44.88***
P450 SCC	1.79
Clusterin	-2.95
AHR	9.25
CDKN2A	-5.46

Table 7-3 Testosterone concentrations to telomere length and %5mc linear models

Beta coefficients are listed. Significance level is denoted by *= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$

Linear model	β coefficient
LRTL1 ~ T1TC	-0.003
LRTL2 ~ T2TC	0.07
BRTL1 ~ T1TC	-0.01*
BRTL2 ~ T2TC	-0.50
CMRTL ~ T1TC	0.007
ERTL ~ T1TC	-0.002
VDRTL ~ T1TC	-0.006
LRTL1 - CMRTL ~ T1TC	-0.02***
LRTL2 - CMRTL ~ T2TC	0.86
LRTL1 - LRTL2 ~ T2TC	0.08
BRTL1 - BRTL2 ~ T2TC	1.82*
%5mc1 ~ T1TC	<0.001
%5mc2 ~ T2TC	-0.30
%5mc1 - %5mc2 ~ T2TC	0.31

Table 7-3 lists the beta coefficients of models using T1TC or T2TC as explanatory variable for telomere lengths or global DNA methylation percentage. BRTL1 was significantly negatively related to T1TC ($F=5.08_{(1,32)}$, $R^2=0.14$, $p=0.03$), as was the difference between LRTL1 and CMRTL ($AIC=115.78$, $P<0.001$). In addition, T2TC was significantly modelled to the difference between BRTL1 and BRTL2 ($F=4.91_{(1,16)}$, $R^2=0.23$, $p=0.04$). Age was added in as an explanatory factor and the significance of the models rechecked. When age was included, T2TC was significantly modelled to BRTL2 ($F=6.31_{(3,16)}$, $R^2=0.54$, $p=0.005$). In addition, with age included T1TC and T2TC were significantly modelled to %5mc1 ($AIC=55.29$, $p<0.001$) and %5mc2 ($AIC=49.31$, $p<0.001$), respectively. HNC and HCC were also added as explanatory variables to explore whether this would alter the significance of the models. By adding HNC1 as an explanatory variable, T1 was significantly modelled to VDRTL ($F=6.59_{(3,30)}$, $R^2=0.40$, $p=0.001$).

7.3.2 Cortisol

Median hair cortisol concentration was 3.46pg/mg (IQR 4.70pg/mg). Cortisol concentrations were log transformed for analyses to improve model residuals. As fur colour has previously been shown to have an impact upon hair cortisol concentration in dogs, fur variables were first checked for differences in cortisol concentration to potentially avoid confounding in further analyses. Due to the range of coat colours, colours were grouped into black, white and other. There was a significant difference in hair cortisol concentration (HCOR) between the three colour groups (Kruskal Wallis test, $p=0.04$, Figure 7.5). Pairwise Wilcoxon rank-sum tests showed there was a significant difference between HCOR in black and white fur ($p=0.03$). Median HCOR in black fur was 5.87pg/mg and 1.58pg/mg in white fur. No significance difference in HCOR was found between short, medium and long hair ($p=0.84$), between coat types ($p=0.78$), or between dogs with single or double coats ($p=0.62$). Median HCOR was higher in low shedding dogs (16.90pg/mg) than high shedding (4.23pg/mg) or moderate shedding (1.65pg/mg) dogs, but the Kruskal Wallis test was not statistically significant ($p=0.15$). There were only 4 dogs in the low shedding group, so small numbers are likely to be the cause of the lack of a significant relationship.

When coat colour was included, age ($p=0.67$), weight ($p=0.61$), HNC2 ($p=0.58$) and HCC2 ($p=0.76$) were not significantly related to HCOR. Breed life expectancy was significantly negatively related to HCOR ($AIC=43.81$, $p=0.004$), and it remained significant after correction for hair colour. HCOR and T2TC were non-significantly negatively correlated (Spearman's $\rho=-0.27$, $p=0.22$). In terms of psychological stress, there was no significant difference in HCOR between dogs purchased directly from a breeder or adopted at an older age ($p=0.67$). Linear models of HCOR to LRTL2, BRTL2 and %5mc2 were not statistically significant ($p=0.17$, $p=0.67$, $p=0.41$, respectively). These models remained non-significant when age was included as an explanatory variable.

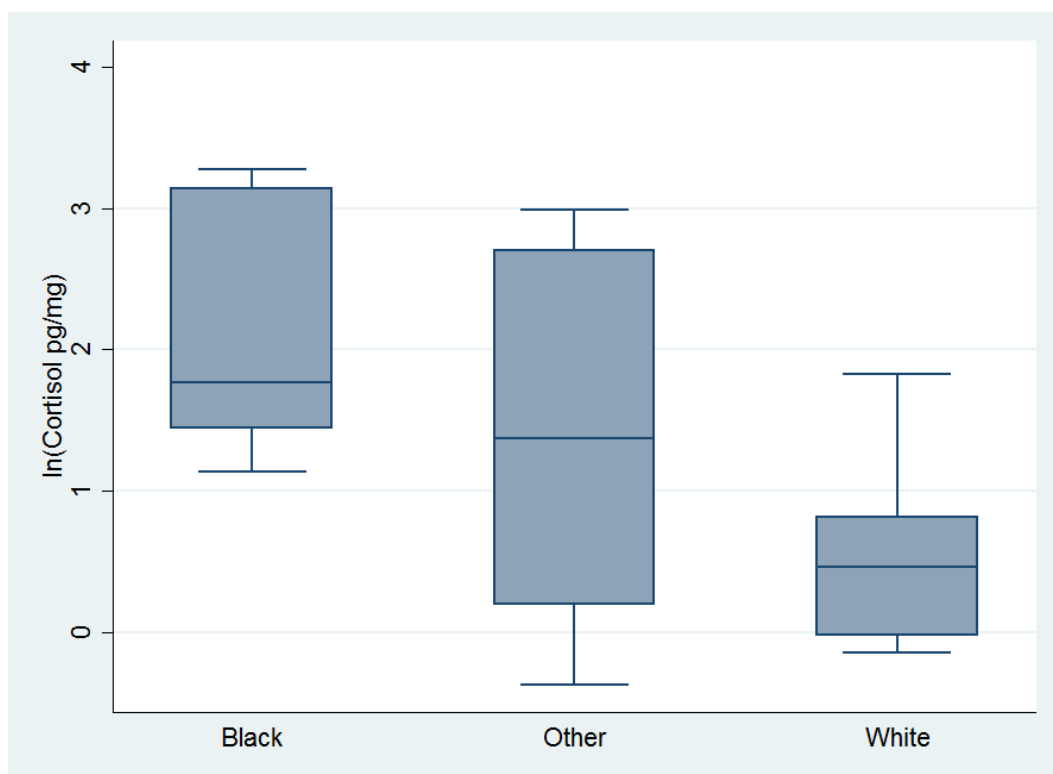


Figure 7.5 Natural log of hair cortisol concentration (pg/mg) by whether cortisol was measured in black, white or another colour of hair.

Cortisol concentration in black hair was significantly greater than in white hair ($p=0.03$). $N=25$ (24 male, 1 female).

7.3.3 Possible factors relating to weight change over the study period

All except 2 of the dogs gained weight above and beyond that expected due to normal growth between the two time points. The percentage weight gains relative to weight at time point 1 (or that expected when fully grown) were calculated. The median percentage weight gain was 15.1% (IQR 16.8%). Factors relating to weight gain were considered. Age of dog was not significantly related to weight gain (Spearman's $\rho=0.15$, $p=0.48$). No significant breed effect could be found. Height did not relate to weight gain (Spearman's $\rho=-0.07$, $p=0.74$), nor did breed cephalic index (Spearman's $\rho=0.05$, $p=0.84$).

Having access to a private garden was not related to weight gain (Wilcoxon rank-sum test, $p=0.98$), nor was the hours of walking per day (Spearman's $\rho=-0.03$, $p=0.90$). Dogs living in urban environments tended to gain more weight than dogs living in rural environments (median percentages: 15.6, 3.32, respectively), but the difference was not significant (Wilcoxon rank-sum test, $p=0.17$). Hair cotinine concentration at time point 2 was significantly positively correlated with percentage weight gain (Spearman's $\rho=0.50$, $p=0.01$, Figure 7.6). Hair nicotine concentration at time point 2 was positively related, however non-significantly, to percentage weight gain (Spearman's $\rho=0.36$, $p=0.08$). The odds ratio of having greater than 20% weight gain between the two time points, if cotinine at time point 2 was positive (based on the ROC generated cut off point from Chapter 3), was 7.5 (95% confidence interval 1.09-51.68) compared to having negative cotinine at time point 2. In addition, weight gain was negatively correlated to T2TC (Spearman's $\rho=-0.41$, $p=0.046$, Figure 7.7). A linear model of T2TC and age to percentage weight gain was statistically significant (AIC=182.88, $p<0.001$).

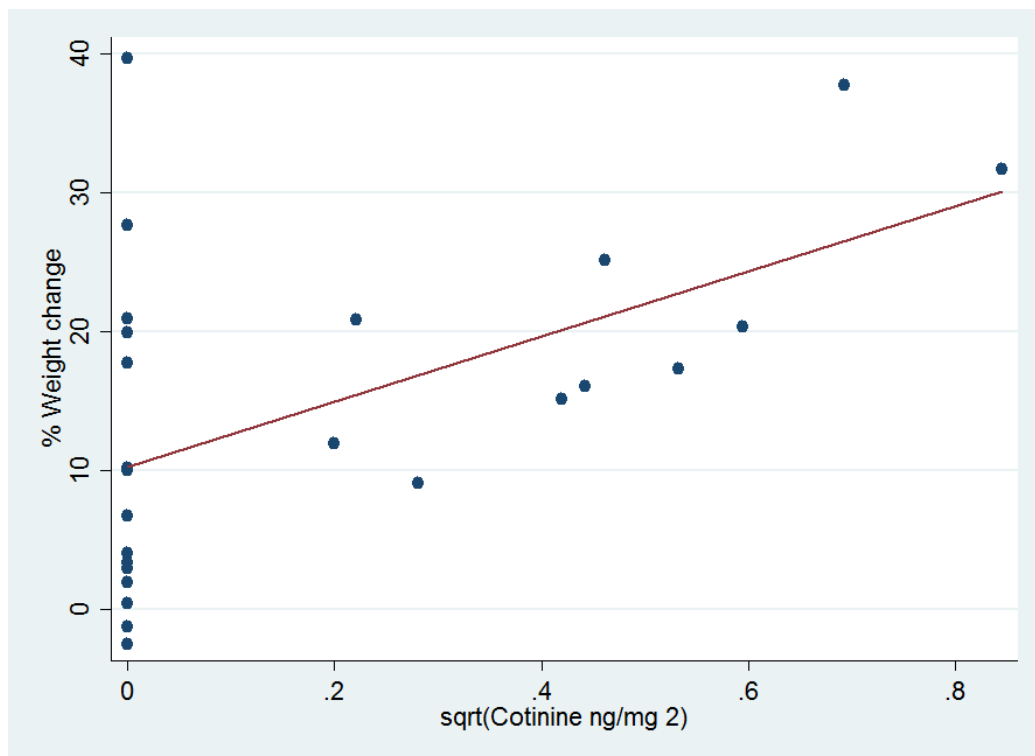


Figure 7.6 Percentage weight change between the two time points and hair cotinine concentration at time point 2

Correlation was statistically significant (Spearman's $\rho=0.50$, $p=0.01$).

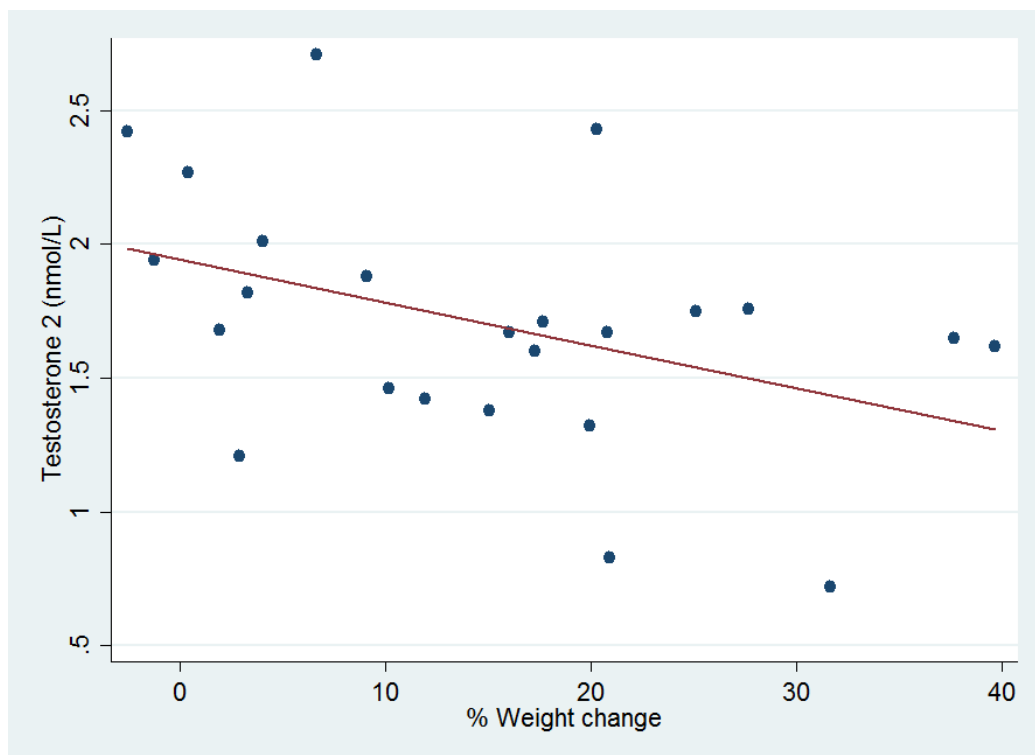


Figure 7.7 Plasma testosterone concentration (nmol/L) and percentage weight change between the two time points

The correlation was statistically significant (Spearman's $\rho=-0.41$, $p=0.046$).

7.4 Discussion

7.4.1 Testosterone

Plasma testosterone concentrations were measured before and after castration. Pre-castration, the concentrations were seen to increase from pre-puberty to puberty. This is in keeping with Mialot *et al.* (1988) who measured testosterone concentrations in blood samples in fox terriers every week from birth until one year of age. They found concentrations remained relatively low for the first 27 weeks of life, then drastically increased. What was unexpected was how high some of the testosterone concentrations were compared to values published in the literature, resulting in concentrations which were above the upper limit of the assay. Multiple studies reviewed showed testosterone concentrations consistently of less than 10ng/ml (e.g. DePalatis, Moore and Falvo, 1978; Urhausen *et al.*, 2009; Albrizio *et al.*, 2013). The majority of published studies involved dogs either kept in kennels or in vivariums. The dogs in this study were in their own homes and would have interacted with other dogs outside. No studies regarding testosterone concentrations in Staffordshire bull terriers could be found. Staffordshire bull terriers had significantly higher plasma testosterone concentrations at time point 1 than the other breeds in this study, so breed selection could play a part in explaining the higher testosterone concentrations found here than in other studies. Having several dogs above the upper limit of the assay limited the analyses and it would be interesting to know how far above the upper limit the concentrations were. It is interesting in particular that Staffordshire bull terriers were the dogs with comparatively high testosterone concentrations, given the numbers of this breed in rescue centres in the UK. Battersea Dogs & Cats Home 2015 annual report stated that Staffordshire bull terriers were the most common breed of dog they rescued, with 1,288 dogs of this breed being taken in by the charity, more than twice the second and third most common breeds: 497 mongrels and 443 Jack Russell terriers (Battersea Dogs and Cats Home, 2016). There is only a limited selection of breeds included in this study, and a more comprehensive examination of breed related differences in concentration of testosterone levels would be of value for both animal welfare purposes and to be instructive for future studies.

As the testosterone concentrations were so high at time point 1, the method of analysis for future studies would need to be improved. In addition, the concentrations of SHBG would be worthwhile measuring, to calculate the concentrations of free testosterone. Mass spectrometry could be employed to measure several proteins at the same time from one sample and could offer a solution to the methodological problems of using a chemiluminescence-based assay. A non-invasive method could be used to obtain the sample required, to avoid the necessity for a Home Office licence. For example, testosterone concentration has been previously measured in dog faeces and was positively correlated with serum testosterone concentration in a study of 22 female beagles (Gudermuth *et al.*, 1998).

A decline in testosterone concentration at older age was not seen in testosterone at time point 1, but this relationship was affected by the presence of prepubescent and pubescent dogs. An inverse weak relationship was seen between age and testosterone concentration at time point 2, when all dogs were post-pubescent. The relationship may have been weak due to the small sample size and lack of spread across the age range, but testosterone concentration could be a potential biomarker of ageing in castrated dogs.

7.4.2 Cortisol

Hair cortisol concentration seemed to be strongly associated with hair colour. Bennett & Hayssen (2010) also observed a difference in cortisol concentration in dogs with hair colour, and demonstrated differences in cortisol concentration within the same animal based on the colour of hair sampled. However, they noted a converse relationship to that found here. They observed lower cortisol concentration in black hair and higher concentration in white hair. Hair cortisol concentrations were also higher in white hair than black hair in cattle (González-de-la-Vara *et al.*, 2011). However, similar to this study, when cortisol concentrations were measured in 2484 human children, the lowest levels were found in blond hair and the highest found in black hair (Rippe *et al.*, 2016). The

observation of higher hair cortisol seen in obese children (Veldhorst *et al.*, 2014) was not replicated in dogs here, possibly due to so many of the dogs gaining more weight than expected between time point 1 and 2, because the relationship was masked by the effects of hair colour on cortisol concentration or differences in physiology between the species. If cortisol concentration had been measured by blood or saliva, an acute stress response may have affected the result. Cortisol response has been shown to be related to telomere length and smoking, as discussed in the chapter introduction, but in order to capture this phenomenon a baseline cortisol reading would still be required. As with testosterone, using a faecal sample may be a way round the issue. The problem would be it would require owner compliance and some may feel that collecting a faecal sample is too unpleasant. Hair and faecal cortisol concentrations were shown to be significantly correlated in study of 29 dogs (Accorsi *et al.*, 2008).

The ELISA used for the measurement of the cortisol concentration may not have produced reliable data. Low concentrations in the samples were measured relative to the standard curve. Ideally, the optical density of the sample wells would fall around the middle of the standard curve, but this was not the case. A more sensitive method may be required to detect the concentrations of cortisol in the fur. However, given the dominant association between hair colour on cortisol concentration, a more sensitive method would be more expensive and time consuming, and yet not necessarily yield any further associations beyond that with colour.

7.4.3 Weight gain

Nearly all dogs in the study gained weight. A large drop in testosterone concentration was observed between the two time points. The drop was large relative to other studies which recorded much lower testosterone concentrations in intact males, as already discussed. If the dogs were fed as prior to their neutering procedure, Root *et al.* (1996) suggested this would lead to weight gain, as the dogs' metabolic rates would have dropped. Diet questions were not

included in the questionnaire, so it is not known to what extent feeding regime contributed compared to ETS exposure or other factors. Body mass index has previously been shown to be related to steps taken by the dog measured using a pedometer (Warren *et al.*, 2011). Daily walking activity was asked about in the questionnaire, but many owners were not forthcoming with this information and the time spent on walks may have been under or over estimated. Daily steps taken could have been measured experimentally using a pedometer, but this would have been beyond the remit of this study. Dogs with positive hair cotinine at time point 2, based on the cut-off point generated in Chapter 3, gained more weight between the two time points. Cotinine represents longer term exposure than nicotine, which is why cotinine may have had a stronger relationship with weight gain than nicotine. Similar findings have been observed in child obesity with parental smoking, as described in the chapter introduction. However, it is not clear why this is. Leptin has a role in regulating body fat mass and has been observed to be at significantly higher concentration in obese dogs than normal dogs (Park *et al.*, 2014). However, in human studies, maternal smoking resulted in decreased leptin concentration in infant cord blood, and in 82 men of mean age 22, smoking was negatively related to leptin concentration (Mantzoros *et al.*, 1997, 1998). Another possibility is IGF-1 level in the dogs exposed to smoking. Pack-year history was positively associated with IGF-1 in a study of 130 adult human subjects aged 30 to 84 years old (Kaklamani *et al.*, 1999). In addition, when 7 adult beagles were experimentally overfed, IGF1 positively correlated with weight gain (Gayet *et al.*, 2004).

7.4.4 Chapter summary

Testosterone and cortisol concentrations were measured in study dogs. Testosterone concentration pre-castration was breed associated and the distribution of the data was affected by limitations of the assay. Testosterone concentration at time point 2 was negatively associated with age, and combined with hair cotinine concentration, was strongly associated with weight gain after castration. Hair cortisol concentrations were related to hair colour but no other factors. Many of the dogs in the study gained weight above that which was

expected for growth. Given the growing prevalence of obesity in dogs, the factors linking increased weight gain with ETS exposure warrant further analyses.

Consultation at the time of neutering surgery may be an ideal point at which to speak to owners about the risks of exposure to ETS for their pet, and owners who do smoke may require extra nutrition advice to manage weight in their pet.

Chapter 8: General Discussion

8.1 Study design and recruitment

8.1.1 Breeds recruited

The initial plan for the study was to only use Staffordshire bull terriers. By keeping to one breed, it was hoped to limit some of the inter-individual variation not explained by age or ETS exposure in both telomeres and further potential biomarkers of ageing. However, to recruit sufficient dogs for the study, the breed pool needed to be widened. Some of the relationships with telomere length observed in this study had not previously been reported. As seen in chapter 4, variables such as cephalic index were shown to be associated with telomere length. This was not anticipated prior to the recruitment of cases and may instruct future studies such that only recruiting one breed might be better, as the sources of inter-individual variation in telomere length in dogs are not currently well described and warrant further investigation. There were issues with recruitment and retention of Staffordshire bull terriers related to factors such as location of the Small Animal Hospital in relation to the place of residence of owners of these dogs. Without private transport, the hospital can be difficult to access and potentially expensive. Owners living some distance away could justify attending for free-of-charge neutering of their animal, but not to return for the follow-up. Owners were offered a free booster vaccination as a 'thank you' for attending the 12-month follow up appointment, but when designing the study, it was not anticipated that several of the Staffordshire bull terriers were not vaccinated, so a preliminary course may have been required rather than a one-off booster. These issues are important for future work, as there is a higher proportion of smokers living in areas with a lower index of deprivation. In addition, these owners benefitted from the offer of free neutering, as cost had previously been a barrier for them, which meant that a wider range of ages of intact dogs were recruited.

8.1.2 Recruitment methods

Local charities and veterinary surgeries were contacted regarding the study, but for various unknown reasons they generally did not assist with recruitment. Some felt that by offering free-of-charge neutering they were being denied business, or they may have been too busy, or felt that they had no incentive to help. Further discussion with such agencies would be beneficial. If sample collection could be moved to a location within easier access for owners without their own transport, potentially this could ease recruitment and retention of owners. The SSPCA were helpful in the provision of testes; however, no history could be gleaned on ETS exposure of these dogs. In addition, age of these dogs was estimated through examination by a veterinary surgeon rather than being a matter of historical record. However, by developing cut-off points in the biomarkers of ETS exposure in hair, the dogs could be sorted into likely exposed and unexposed groups from a sample of their hair. In which case, further exploratory data could be generated prior to recruiting owners and dogs for future studies.

While retention was an issue, it would be interesting to follow dogs long-term to see whether changes in ageing biomarkers resulted in earlier onset of diseases of ageing. However, such a study would take a long time and potentially be expensive. If asking about ETS exposure became part of routine veterinary history taking, potentially a study on veterinary databases could examine prevalence of diseases in ETS exposed and non-ETS exposed and at what age they occurred. If diseases of ageing were demonstrated to occur at a younger age in ETS-exposed dogs, this could be the platform to justify a large long-term biomarker of ageing study in pet dogs.

8.1.3 Limitations

As one person conducted the owner questionnaires, arranged sample collection, performed experimental procedures and data analyses, there was a lack of blinding in this study. One of the few experiments not conducted by the author

was the measurement of hair nicotine and cotinine concentrations. These measurements were then used as objective measures of exposure in subsequent chapters. However, ideally, the person performing the laboratory work would be completely blind to the exposure status of the dog. Unfortunately, there was not a sufficient budget available for such a person to be available.

8.2 Biomarkers of ETS exposure

8.2.1 Use of nicotine and cotinine

As seen in Chapter 3, hair nicotine concentration reflected the ETS exposure of the dogs well. In addition, a decrease in hair nicotine concentration was seen when in 3 of 4 owners who gave up smoking between the two time points. A drawback of using nicotine and cotinine as biomarkers of exposure, is that these compounds are not carcinogenic. As discussed in Chapter 1, the carcinogenic compounds in tobacco smoke are present at a lower concentration than nicotine, so there may be issues with their measurement. A recent study evaluated the measurement of tobacco specific nitrosamines in hair of non-smokers, and found that NNK showed promise as a biomarker of exposure (Pérez-Ortuño *et al.*, 2016). Pilot data could be generated to see if it would be possible to quantify NNK in hair of ETS exposed dogs, and if this relates to future cancer diagnosis.

8.2.2 Aid to smoking cessation

As discussed in chapter 3, if smoking owners could be shown their dog's hair nicotine concentration before and after giving up smoking, this could be an incentive to help them maintain a quit attempt. Figures from surveys of Scottish smokers discussed in Chapter 1 imply that while around two-thirds of smokers wish to stop smoking, long term cessation can be an issue. However, an exception to this plan would be the roll-your-own smokers, many of whom felt their habit did not cause harm to those around them. Educating these owners regarding the harmful effects of ETS with their habits would certainly be a challenge and may

need to come from elsewhere, though awareness of adverse effects on pets could be a contributory incentive for some.

8.3 Experimental procedures

A qPCR protocol was developed for the measurement of telomeres. While the inter-assay variability of the protocol was relatively low compared to those published elsewhere, Southern blot is regarded as the gold standard for telomere measurement. In addition, qPCR only provides an average length of telomeres, whereas Southern blotting procedures can be used to look at the proportion of short telomeres, which could be related to cellular senescence. However, the inter-assay variation of the Southern blot procedures in this thesis was high. Further work would need to be undertaken to reduce this variability. Furthermore, some of the procedures could be streamlined to avoid resource waste. For example, columns could be used such as AllPrep by Qiagen (Dusseldorf, Germany) to simultaneously extract DNA, RNA and protein from the same sample. This would enable telomere measurement and mRNA expression levels of CDKN2A, for example, from the same sample. In addition, different brands and methods of DNA extraction were used in this thesis. As was discussed in chapter 4, variation in telomere length has been related to the use of different extraction methods, so one method or brand alone should be used throughout a study.

Further limitations of assays used has been discussed in the relevant chapters.

8.4 Tissues used for analyses

Where possible, tissues which were either minimally invasive to collect or would otherwise be discarded, were used. By using such tissues, samples can be collected from companion animals and the use of laboratory animals can be avoided. As described in chapter one, biological ageing in dogs is more similar to humans than that of mice, so results could be more applicable to humans. In

addition, for a subsection of the population, the use of experimental animals is not acceptable morally and samples such as these could have greater ethical acceptance. However, there were some issues which arose when using these samples, which will be further discussed here.

8.4.1 mRNA expression in testes

mRNA expression levels of various potential biomarkers of ageing were measured in testes in chapter 6. While the testes were used because they were snap-frozen immediately after castration enabling extraction of good quality RNA, and they provide a large amount of tissue which would otherwise be discarded, several of the gene expressions appeared to be associated with puberty status. Whether the same patterns of expression would occur in other tissues is unknown. In addition, a dog can only be castrated once, so using this tissue does not offer the potential for longitudinal measures of gene expression levels. In addition, many owners opt to castrate their dogs at around 6 months of age. There appeared to be an association between mRNA expression levels of some of the genes and changes related to growth or puberty. It would be interesting to measure gene expression levels of these markers in blood; however, collection of blood has practical, ethical and legal issues in the UK, for which a Home Office licence is required (unless the blood is surplus to requirements from clinically directed sampling). Spare blood samples obtained from the Veterinary Diagnostic Service were only available after several days' post sampling. Experiments would need to be conducted as to how long RNA in a blood sample would be stable for to assess whether spare blood could be used for such experiments.

8.4.2 Oral telomere length

Buccal and saliva telomere length appeared to be associated with neutered status in males. An explanation for this could not be found. Data from chapter 4 and 5 show that entire males often have shorter oral telomere length than castrated males. In addition, chapter 7 data showed an association between buccal

telomere length and testosterone concentration, reinforcing the potential link between buccal telomere length and neuter status. Possibly, there is something in the intact male saliva which causes DNA degradation. As telomeres are at the ends of chromosomes, they are at greater risk of degradation than the rest of the chromosome. Without examination of the saliva of intact and castrated males it is difficult to speculate. Even with such samples, it would be difficult to know what to look for, possibly digestive enzymes, but this would be entirely speculative. Another problem with the use of oral DNA in some of the dogs was aggression. If the dog did not like the person collecting the sample going near its mouth, this left the person collecting the sample at risk of a bite injury. Some dogs were swabbed through a muzzle, but if they were particularly stressed this was a rather difficult task, which was also questionable ethically.

One further use for buccal DNA could be assessment of methylation. Buccal DNA has been used for this purpose in human studies (e.g. Kaminsky *et al.*, 2009). Given that changes in 5-mc could be demonstrated at time point 1 with both age and ETS exposure, it would be interesting to see if these findings could be replicated in a non-invasive sample.

8.4.3 Hair sampling

While hair was successfully employed for the measurement of ETS biomarkers, cortisol concentration varied with colour of hair. In addition, some of the dogs were nervous of clippers used to collect the hair sample. As mentioned in chapter 7, in future work steroid hormones could be measured in faecal samples. A mass spectrometry protocol could be developed which measured hormones and ETS biomarkers from one sample. However, hair nicotine was shown to be a good representative measure of the dog's exposure and faecal ETS biomarkers may not be as good of a reflection.

8.5 Issues related to neutering

Nearly all the dogs in the study gained weight as discussed in chapter 7. Dogs with a positive cotinine measure at time point 2 tended to have a higher percentage weight gain. Potentially, this warrants further discussions with the owners of dogs at the time of neutering. While the dogs are at lower risk of diseases such as pyometra in females and testicular tumours in males, there could be a risk of harming the dogs due to excessive weight gain causing diseases related to obesity. In addition, many of the dogs in non-smoking homes gained weight as well, so all owners may benefit from education regarding energy intake requirements after neutering, not just those in smoking homes.

Staffordshire bull terriers were found to have increased testosterone concentrations at time point 1 compared to other breeds. This was found in conjunction with increase 17βHSD mRNA expression and decreased CDKN2A mRNA expression in this breed in chapter 6. Testosterone can be related to unwanted behaviours in dogs such as aggression, sexual behaviours and roaming. This breed often has a negative reputation and it would be interesting to evaluate more fully whether testosterone concentration is a key part of this story. Two of the Staffordshire bull terriers were relinquished by their owners during the study due to aggression. However, this was, of course, after they were castrated, so there may well be a learnt behavioural component to this potentially complex issue. One Staffordshire bull terrier had a lower testosterone concentration than the others. He had recently been adopted and had previously been maltreated. As this was an isolated example, it is not known whether he is an exception in terms of his breed or if owner treatment influenced testosterone concentration in this dog. Further studies are certainly warranted around this for the welfare of this breed. In addition, a wider range of breed testosterone levels would be useful to assess whether any other breeds have particularly high testosterone concentrations. This could also influence the selection of dogs for use in future studies examining gene expression in testes, to avoid breed confounding issues.

8.6 Further potential areas of investigation

8.6.1 Biomarkers of ETS exposure

As already mentioned, the measurement of NNK concentration in dog hair could be trialled as a biomarker of ETS exposure. As NNK is a carcinogen, if it is possible to quantify it in dog hair, it could be measured in oncology cases and a control group to see if concentration of NNK in dog hair is a risk factor for cancer diagnosis. In the additional analyses section for Chapter 3, it was observed that there was a potential sex effect on nicotine metabolism. Another possible source of variation is genetic variation of P450 2A6. Genetic variations exist in humans (Malaiyandi, Sellers and Tyndale, 2005) and polymorphisms of other cytochrome P450 enzymes exist in dogs (e.g. Aretz and Geyer, 2011). Therefore, it would be useful to investigate if polymorphisms exist in P450 2A6 which could impact on metabolism in different breeds.

8.6.2 Telomere length measurement

A potential way to measure telomere length in dogs in future studies could be DNA sequencing. This would avoid problems encountered such as the miniature schnauzers having very low telomere C_T values when using qPCR, and DNA quality ratios being related to C_T values. While the price of this technique was too high for use in this study, prices are decreasing and in the future sequencing may be a viable option for this type of study.

While there were many possible sources of inter-individual differences in telomere length observed, a significant relationship was demonstrated between leukocyte telomere length and ETS exposure. However, unlike in the samples used in Chapter 4, there was no significant relationship observed between leukocyte telomere length and age in the ETS study dogs. A study utilising one breed of a

wider range of ages could be used to see if there is a difference in age-related changes in telomere length with ETS exposure.

8.6.3 CDKN2A expression

CDKN2A expression was significantly related to ETS exposure, but not age. This could be due to the use of testes and the age distribution of the cohort. A study of CDKN2A in a cohort using a different tissue could be trialled. In addition, a different marker of senescence, such as beta-galactosidase, could be used to provide further evidence for the presence of senescent cells.

8.6.4 DNA methylation

Global DNA methylation was related to ETS exposure. As discussed in Chapter 1, a study earlier this year used the methylation status of selected CpGs to generate an “epigenetic age” in dogs (Thompson *et al.*, 2017). A method such as used by Thompson *et al* could be utilised to see if dogs exposed to ETS have an older DNA methylation age than those not exposed.

8.7 Overall conclusions

This study has provided a large amount of pilot data. The numbers involved were small, but many avenues for further work have been generated. This study has also demonstrated the use of a One Health approach to science, with the results of such an approach potentially benefitting both humans and animals.

The study has shown:

- In relation to published literature at the time of the study's inception, greater insight into the methods available for studying markers of ageing in dogs;
- Clear evidence of an effect of ETS on some markers of ageing in verifiably exposed dogs;
- Evidence of interesting and potentially important relationships between markers of ageing and testosterone concentration in male dogs that appears to be variable among breeds;
- Some evidence that changes that occur in male dogs as they reach sexual maturity may be more complex and far reaching than the physiological and behavioural changes already known

Appendix 1: Study information for owners

ASSESSING THE EFFECTS OF ENVIRONMENTAL TOBACCO SMOKE ON CANINE TELOMERE LENGTH & REPRODUCTIVE FUNCTION

STUDY INFORMATION FOR OWNERS

Thank you very much for taking an interest in participating in this study which is aiming to look at the effects of passive smoking on pet dogs. We will be particularly looking at the effect on a certain part of your pet's DNA (the telomere), hormone levels and the cells in their testicles. We will do this by neutering your pet and studying tissues collected at this point, and performing follow up health checks and tests over the course of a year.

We are looking for male pet dogs to take part who have not been neutered previously- they will be neutered free of charge as part of the study. The dogs can be of any age. The only breeds excluded are those which are toy and giant sizes, due to the way the telomere behaves in these breeds, and non-shedding breeds such as poodles. We would prefer the dogs not to have any major illnesses, but we can discuss suitability of your dog to take part depending on what the illness is.

The study will be comparing dogs from smoking and non-smoking households, so you are welcome to take part whether or not you and/or another member of your household smoke. The levels of nicotine the dog has been exposed to will be measured by taking a small sample of fur at intervals. We will ask you specific questions on the days that fur samples are taken so we can gather information regarding the dog's exposure and other factors that may affect DNA length. We will make use of the information about your dog and that of others enrolled in our study to learn more about factors that may be related to DNA length in dogs. Reports of our findings will be based on trends in groups of dogs and will not identify individuals.

You will be asked to participate in the study for one year. At the initial assessment, your dog will have a check by a vet, blood tests, cheek DNA samples and a fur sample taken, and you will be asked various questions about your pet's health and exposure to tobacco smoke. If the dog is confirmed to be suitable for the study, a date will then be arranged for them to be

neutered. We will examine the dog's testicles and the surrounding tissues removed routinely at surgery. All post-operative care required will be provided by the Small Animal Hospital and we will provide pain relief for you to administer to your dog at home. You will be requested to return at 12 months after your dog is neutered for follow up health checks, cheek DNA tests and fur sampling. During the year, I will contact you at 3 monthly intervals to check whether the smoking status of your household has changed and to see if your pet has had any health problems.

As a thank you for participating, your dog will be given a free vaccination when you have completed the study (the exact date the vaccine is provided will depend on at what point your dog is in its vaccination programme). You will be given two lots of wormers free of charge for allowing me to contact you 3-monthly. Unfortunately, we will not be able to cover transport costs for your attendance at our clinic.

If you would like to enquire further regarding the study or would like to sign up, please contact:

Natalie Hutchinson n.hutchinson.1@research.gla.ac.uk

Appendix 2: Owner time line information sheet

EFFECTS OF ENVIRONMENTAL TOBACCO SMOKE ON CANINE TELOMERE LENGTH & REPRODUCTIVE FUNCTION

Study Number..... Name of Dog.....

This sheet provides a place to record your appointment dates and gives you an idea of what will happen at each session.

NB- You are not charged money for any of these appointments or services. If you are asked to pay at any point, please do not do so and contact Natalie Hutchinson or Clare Knottenbelt who will remedy this situation.

Initial assessment:

Date:

Time:

What will happen at this appointment:

This is a meet-and-greet where you can find out more about the project and we can check whether your dog is definitely a suitable candidate. You will be asked to complete a questionnaire about your dog and the environment they are kept in- this will take around 15 minutes to fill in. You will be asked to sign a study consent form so we will be able to use the information you provide us for the project.

Your dog will be examined by a vet and a blood sample taken for routine tests to ensure he is fit and well for surgery. You will be notified immediately by telephone if there is any abnormality found in the blood tests when we have the results. In order to take the blood sample, we will need to clip some fur from your dog's neck. This fur clipping will be tested for nicotine levels.

Neutering:**Date:****Admission Time:**

What will happen at this appointment:

If you are happy to proceed with the study after the initial assessment, you will be given a date for your dog to be admitted to the Small Animal Hospital and undergo a neutering procedure. This is a surgical procedure and therefore a member of hospital staff will go through the necessary information and provide aftercare advice for you to follow. You will be asked to sign a separate consent form for surgery. You will be given medication to give to your dog at home for pain relief. You will be kept informed over the course of the day with how your dog is getting on and all being well he will be ready for collection later on the same day.

During the surgery, we will take a cheek swab and retain the tissues removed during the operation for our study.

You will need to come back for your dog's stitches to be removed at 7-10 days after this day.

Removal of stitches:**Date:****Time:****3/6/9 month checks (by phone or email):**

What will happen:

You will be asked to complete a short questionnaire detailing any significant events that have occurred to your dog since we last saw you and any changes in the environment your dog lives in. This can be done easily via phone or email.

As a thank you for completing the questionnaire, you will be provided with two lots of worming medication for your dog free of charge when you return for the 12 month appointment.

12 month check (at Small Animal Hospital):

Date:

Time:

Your dog will have a vet check and a blood sample taken. This will again involve clipping fur from the neck region. Routine blood tests will be performed on the sample and you and your local vet will be notified of the results. We will take a cheek swab as well. You will be asked to complete a final short questionnaire.

As a thank you for attending, arrangements will be made for your dog to be vaccinated free of charge.

If you have any problems with attending one of your appointments, please contact Natalie Hutchinson on 0141 330 8578 or by email on n.hutchinson.1@research.gla.ac.uk

Or the Small Animal Hospital directly on 0141 330 5848 (please mention you are part of Clare Knottenbelt's passive smoking dog study when speaking to reception so appropriate appointments can be arranged)

Appendix 3: Consent form

School of Veterinary Medicine
Faculty of Veterinary and Life Sciences
University of Glasgow
464 Bearsden Road
Glasgow
G61 1QH

ASSESSING THE EFFECTS OF ENVIRONMENTAL TOBACCO SMOKE ON CANINE TELOMERE LENGTH & REPRODUCTIVE FUNCTION

STUDY CONSENT FORM

Project Number:

Owner:

Dog Name:

Address:

Age:

Sex:

Date:

I, the owner, or agent of the above animal, hereby give my consent for the above animal to be included in this study.

I understand that as part of the study the dog will be neutered and, as such, will be administered anaesthetic drugs, have surgery and require post-operative medication and care. I understand that there is a small risk of anaesthetic complications, bleeding and infection with such a procedure. I understand that to ensure my dog is fit to undergo such a procedure, pre-operative routine blood tests will be performed. I give my permission for the retention of the dog's reproductive organs which will be removed at neutering. At the end of one year following the neutering procedure I agree for my dog to have further blood testing as part of a routine health check. I also give my permission for a mouth swab to be taken at both these points.

All samples, including swab from the mouth, semen, fur, testicles with surrounding tissues, and bloods taken will be retained for this study and other future research projects.

I understand that I will be asked to complete questionnaires detailing the exposure to tobacco smoke that this dog has experienced on a regular basis. Fur clippings will be taken from the animal's neck and tested for nicotine levels to confirm tobacco smoke exposure level.

I understand that my dog will be allocated a project number and that all documents relating to this study will therefore remain anonymous, however the information may be accessed by staff at the Small Animal Hospital in order to provide appropriate care for the dog.

I understand that it is my right to withdraw from the study at any time and that the participation of the dog in the study may be terminated early in the case of issues such as the dog developing a major illness during the study period.

Signed.....Date.....

Appendix 4: Letter to owner's local veterinary surgeon



School of Veterinary Medicine
College of Medical, Veterinary and Life Sciences
University of Glasgow
464 Bearsden Road
Glasgow
G61 1QH

27/08/2017

**ASSESSING THE EFFECTS OF ENVIRONMENTAL TOBACCO SMOKE ON
CANINE TELOMERE LENGTH & REPRODUCTIVE FUNCTION**

Dear

Re:

The above named dog has been enrolled in our studies, “Assessing the Effects of Environmental Tobacco Smoke on Canine *Telomere Length*” and “Assessing the Effects of Environmental Tobacco Smoke on Canine *Reproductive Function*”. The owner has granted me permission to contact you in inform you of their involvement in the study, and to communicate to you clinical information gathered during the study period regarding their animal.

We are recruiting male pet dogs from the Glasgow area, collecting various samples from them to perform DNA extractions on and thus measure telomere length. We

will be working with 40 dogs in all, dividing them into exposure groups based on both information obtained from the owner and by performing mass spectrometry analysis on fur samples. All of the appointments for the study will take place at the University of Glasgow Small Animal Hospital and the clinical care will be overall supervised by Clare Knottenbelt, the Clinical Director of the hospital.

In order to collect the initial samples, the dog will be neutered following a pre-operative assessment. At the pre-operative assessment, the dog will be examined by one of the vets at the Small Animal Hospital and blood samples for routine haematology and biochemistry tests taken as a means of health screening, the results of which will be made available to you. If the dog is deemed suitable for the study, they will be admitted to the Small Animal Hospital soon after initial assessment for the neutering procedure. We will retain the tissues removed at surgery for our study, and testicles and ovaries will then also be used for the collaborative study on reproductive function. Post-operative care and medication will be provided by the hospital.

The dog will then be followed up for a period of 12 months. At 3, 6 and 9 months the owner will be contacted by telephone to check if there has been any change in their smoking or in the health of the dog. At 12 months, the owner will be asked to bring their dog back to the Small Animal Hospital, when the dog will be examined by one of the vets and also have a further routine blood test. In order to encourage owners to stay on the study, free wormers will be provided for completing questionnaires and a free vaccination will be given for attending the 12 month appointment.

The owner will not be charged for any of the services provided related to the study, including the neutering procedure. We hope you do not feel that we are encroaching on services you would wish to provide for this animal. Several of the dogs we are enrolling will be through charitable agencies who would normally partly or wholly financially contribute to the neutering of these animals, so we hope to ease their burden in a small way.

If you know of any intact male dogs you would like to be considered for the study, or have any other questions, please do feel free to contact myself or Clare Knottenbelt using the details below. The surest contact to use is my email address (n.hutchinson.1@research.gla.ac.uk). Please note, you may have heard previously we are recruiting only Staffordshire Bull Terriers, but we have now widened the study to all breeds, bar toy and giant size and non-shedding breeds.

Yours sincerely

Natalie Hutchinson, PhD Student

353 Jarrett Building
464 Bearsden Road
University of Glasgow
G61 1QH
Office: 0141 3308578
Email: n.hutchinson.1@research.gla.ac.uk

Clare Knottenbelt
c/o Small Animal Hospital
Office: 0141 3307459
Email: clare.knottenbelt@glasgow.ac.uk

Appendix 5: Consent form to contact local veterinary surgeon

**ASSESSING THE EFFECTS OF ENVIRONMENTAL TOBACCO SMOKE ON
CANINE TELOMERE LENGTH & REPRODUCTIVE FUNCTION**

PARTICIPANT LOCAL VET

Participant Number:

Dog Name:

Local Vet Name/Practice:

I, the undersigned, hereby do/do not give permission for my local vet to be informed of my dog's participation in this study. I do/do not given permission for details regarding procedures undertaken and test results obtained to be communicated to this vet.

Signed

Date.....

Appendix 6: Questionnaire

Assessing the Effects of Environmental Tobacco
Smoke on Canine Telomere Length:
Dog Fact Sheet: 1

Section 1: General information about your dog

1. How old is your dog? Years Months

→ Is this age approximate ☐ Approximate ☐ Accurate

2. How long have you owned your dog? Years Months

3. How did you come to own your dog?

☐ Born into my home- *go to section 2*

☐ Purchased as puppy- *go to section 2*

☐ Rehomed e.g. from a charity, from an acquaintance- *continue to question 4*

4. If you rehomed your dog, where was this from?

5. Do you know anything about the dog's life history prior to rehoming?

☐ Yes ☐ No- *go to section 2*

6. If you know about your dog's life history prior to rehoming, could you please elaborate?
Paying particular attention to any major events, illness or injury, ill treatment and, for the relevance of our study, whether the dog was previously in a smoking household

Section 2: your dog's health

<p>7. Has your dog ever been treated for any of the following medical problems? Please give further details on the nature of the problem, treatments given and the date the problem was diagnosed.</p>	<p>Condition</p> <p><input type="checkbox"/> Cancer</p> <p><input type="checkbox"/> Epilepsy/seizures/fits</p> <p><input type="checkbox"/> Anaemia/other blood disorder</p> <p><input type="checkbox"/> Heart disease</p> <p><input type="checkbox"/> Lung disease</p> <p><input type="checkbox"/> Kidney disease</p> <p><input type="checkbox"/> Chronic diarrhoea/ other gut problem</p> <p><input type="checkbox"/> Liver disease</p> <p><input type="checkbox"/> Diabetes</p> <p><input type="checkbox"/> Allergies</p> <p><input type="checkbox"/> Skin complaints</p> <p><input type="checkbox"/> Arthritis/ other joint problem</p>
---	--

→ Please give details of illnesses and list any other conditions your dog has experienced that are not named here

8. Has your dog had any surgical procedures in the past?

☐

Yes

☐

No

☐

Don't know

→ If Yes, please list

9. If your dog is female, has she had any puppies previously?

☐

N/A (dog is male)

☐

Yes

☐

No

☐

Don't know

→ If yes, how many litters has she had?

10. Does your dog receive any regular medications (including creams, drops, etc.)?

☐

Yes

☐

No

→ If yes, please specify

11. When was your dog last vaccinated?

12. How many times have you wormed your dog in the past 6 months?

☐

None (skip to question 14)

☐

Once

☐

Twice

☐

More than twice

13. If you have used wormers, what was the method of administration? (tick all that apply)

☐

Oral (tablet/mixed in with food)

☐

Topical (Spot On)

14. How many times have you used products against fleas and/or ticks on your dog in the past 6 months?

- ☐ None (skip to question 16)
- ☐ Once
- ☐ Twice
- ☐ More than twice

15. If you have used flea or tick treatments, what was the method of administration? (tick all that apply)

- ☐ Oral
- ☐ Spot On formulation
- ☐ Spray
- ☐ Shampoo
- ☐ Collar
- ☐ Other

→ If other, please specify

Section 3: your dog's grooming regime

16. Are there any other dogs in your household?	<input type="checkbox"/> Yes	<input type="checkbox"/> No
→ If yes, does this dog groom (lick) any of these other dogs?	<input type="checkbox"/> Yes	<input type="checkbox"/> No

17. How often do you bathe your dog (i.e. clean with soap & water) or use a grooming service for this?	<input type="checkbox"/> At least once per week
	<input type="checkbox"/> At least once per month
	<input type="checkbox"/> Every 2-3 months
	<input type="checkbox"/> Less often than 2-3 monthly
	<input type="checkbox"/> Never

18. How often do you brush your dog's fur?	<input type="checkbox"/> More than once per week
	<input type="checkbox"/> Once per week
	<input type="checkbox"/> At least once per month
	<input type="checkbox"/> Less than once per month
	<input type="checkbox"/> Never

19. How often do you brush your dog's teeth?	<input type="checkbox"/> More than once per week
	<input type="checkbox"/> Once per week
	<input type="checkbox"/> At least once per month
	<input type="checkbox"/> Less than once per month
	<input type="checkbox"/> Never

Section 4: your dog's environment

20. Thinking of an average day, what routine would your dog have? → How many hours inside? Of these hours inside, how many are spent in a room where smoking takes place? → How many hours outside? Of these hours outside, how many are spent in the company of someone who may be smoking? → How many walks does the dog go on & what duration are these?	<input type="text"/>	<input type="text"/>	Hours inside
	<input type="text"/>	<input type="text"/>	Hours inside with smoker
	<input type="text"/>	<input type="text"/>	Hours outside
	<input type="text"/>	<input type="text"/>	Hours outside with smoker
	<input type="text"/>		Time being walked
<div>Further detail:</div> <div style="border: 1px solid black; height: 250px; width: 100%;"></div>			

21. Does your dog have access to a private garden?	<input type="checkbox"/> Yes <input type="checkbox"/> No- go to ques
---	---

22. If your dog has access to a garden, are any of the following used there (tick all that apply) → If Other, please specify	<input type="checkbox"/> Weed killer
	<input type="checkbox"/> Slug/snail killer
	<input type="checkbox"/> Ant killer or other insect killer
	<input type="checkbox"/> Fertiliser e.g. on lawn, on plants
	<input type="checkbox"/> Other chemical product in garden
	<input type="text"/>

23. Are any pest control products used in the indoor environment to which your dog has access?
- ☐ Yes
- ☐ No

→ If yes, please specify

24. Has your dog ever lived in the same household as a smoker?
- ☐ Yes
- ☐ No/not that I'm aware of

25. Has your dog been in regular contact with a smoker in the past 3 months?
- ☐ Yes
- ☐ No

(NB- regular contacts includes household members and people interacted with on a DAILY basis outside the home)

IF YOU HAVE ANSWERED "NO" TO QUESTION 26 YOU MAY FINISH THE QUESTIONNAIRE HERE. THANK YOU. IF YOU ANSWERED "YES" TO QUESTION 25, PLEASE COMPLETE THE REMAINING QUESTIONS.

26. For how long has your dog been in regular contact with a smoker?
- ☐ For entire length of ownership
- ☐ 0-3 months
- ☐ Longer than 3 months but less than one year
- ☐ 1-2 years
- ☐ 3-4 years
- ☐ 5+ years

Space for added details provided by owner. For example, intermittent exposure with periods of giving up smoking, changes in who is at home.

27. When your dog is in contact with tobacco smoke where does this take place? Please indicate by circling for each of the following places if this contact is regularly (daily), occasionally (weekly or less) or never.

During close contact with a smoker

In pet's home	Regularly/ Occasionally/ Never
During car travel	Regularly/ Occasionally/ Never
In friend's or family's home	Regularly/ Occasionally/ Never
Outdoors	Regularly/ Occasionally/ Never
	Regularly/ Occasionally/ Never

28. How many smokers does your dog regularly come into contact with:

a) **Within their house of residence**

--	--

b) **Outside their house of residence**

--	--

29. For all smokers included in the answer to question 29, please list how many cigarettes and other tobacco products they smoke per day on average.

Smoker 1: Hours per day with dog

Cigarettes (including roll up):

☐

1-20

☐

21-40

☐

41+

Other tobacco products:

☐

Pipe

→ If yes, length of time pipe smoked per day

☐

Cigars

→ if yes, number of cigars

Smoker 2: Hours per day with dog

Cigarettes (including roll up):

☐

1-20

☐

21-40

☐

41+

Other tobacco products:

☐

Pipe

→ If yes, length of time pipe smoked per day

☐

Cigars

→ if yes, number of cigars

Smoker 3: Hours per day with dog

Cigarettes (including roll up):

☐

1-20

☐

21-40

☐

41+

Other tobacco products:

☐

Pipe

→ If yes, length of time pipe smoked per day

☐

Cigars

→ if yes, number of cigars

Smoker 4: Hours per day with dog

Cigarettes (including roll up):

☐ 1-20

☐ 21-40

☐ 41+

Other tobacco products:

☐ Pipe

→ If yes, length of time pipe smoked per day

☐ Cigars

→ if yes, number of cigars

Smoker 5: Hours per day with dog

Cigarettes (including roll up):

☐ 1-20

☐ 21-40

☐ 41+

Other tobacco products:

☐ Pipe

→ If yes, length of time pipe smoked per day

☐ Cigars

→ if yes, number of cigars

Smoker 6: Hours per day with dog

Cigarettes (including roll up):

☐ 1-20

☐ 21-40

☐ 41+

Other tobacco products:

☐ Pipe

→ If yes, length of time pipe smoked per day

☐ Cigars

→ if yes, number of cigars

Thank you very much for completing the fact sheet. Your help with our study is greatly appreciated. If you wish to give feedback on the questionnaire, please do so to a study representative

Appendix 7: Chapter 3 additional analysis

Variables associated with hair nicotine and cotinine

Factors related to HNC and HCC in non-ETS exposed dogs

In the dogs that were not exposed to ETS at home, the factors which were associated with their HNC and HCC measures were investigated, to see if there were any factors which could explain why there were no dogs with a HNC of 0ng/mg. Given the small numbers involved, it was likely that statistically significant relationships would not be found, but informative trends may have been seen. In addition, as previously stated, HNC1 was not statistically significantly increased in group 3 dogs compared to group 2 dogs (Wilcoxon rank-sum test, $p=0.22$), nor was HCC1 increased in group 3 compared to group 2 ($p=0.58$). The non-significant difference in HNC1 may be due to the sample size as the groups visually appear different on the box and whisker plots. Therefore, analyses were conducted to explore whether any other variables could be associated with HNC1 and HCC1 other than smoking not occurring indoors which could be corrected for in analyses.

Time point 1 measures were focussed on, due to the higher number of dogs included compared to the number at time point 2. Firstly, dog characteristics were considered. No meaningful analyses could be conducted into breed or sex effects due to the wide variety of breeds and small number of females. HNC1 non-significantly increased with dog age ($AIC=71.67$, $p=0.23$). The relationship was less strong between HCC1 and age ($AIC=-26.97$, $p=0.53$). The linear model of dog weight to HNC1 was statistically significant ($AIC=68.67$, $p=0.04$), with weight being positively related to HNC1. However, the linear model of breed height to HNC1 was not statistically significant ($AIC=71.77$, $p=0.24$). In addition, when HCC1 was examined, the linear model of dog weight to HCC1 was highly significant ($AIC=-30.08$, $p<0.001$), with weight being positively associated with HCC1. Unlike HNC1, breed height was significantly related to HCC1 ($AIC=-27.14$, $p=0.001$), with taller dogs having an increased HCC1. Breed cephalic index was non-significantly related to HNC1 ($AIC=60.40$, $p=0.23$), but significantly positively associated with HCC1 ($AIC=-24.61$, $p<0.001$). Coat-related factors were also considered. A Kruskal-Wallis test of fur length (short, medium, long) by HNC1 was statistically

significant ($p=0.04$); however, no pairwise Wilcoxon-rank sum tests were statistically significant after Holm p value correction, with the difference between medium and short hair being close to significance ($p=0.06$). These findings regarding coat length were replicated with HCC1. Single coat dogs had non-significantly increased HNC1 (Wilcoxon-rank sum test, $p=0.08$) and had borderline statistically significant increased HCC1 compared to double coat dogs ($p=0.045$). Rate of shedding (low, moderate, high) and coat type (silky, smooth, wirey) did not impact HNC1 ($p=0.57$, $p=0.76$ respectively). HNC1 was not significantly different based on hair colour ($p=0.34$). In addition, rate of shedding, coat colour and coat type were not related to HCC1 ($p=0.57$, $p=0.41$, $p=0.69$, respectively). Regarding owner treatment of the dog's coat, no significant differences in HNC1 or HCC1 were found based on regularity of brushing (HNC1: $p=0.45$; HCC1: $p=0.43$) or bathing (HNC1: $p=0.76$; HCC1: $p=0.95$).

Factors relating to the dogs' environments were also considered. The linear model of index of deprivation to HNC1 was not statistically significant ($AIC=71.75$, $p=0.24$). However, a significant relationship existed between index of deprivation and HCC1, with the linear model of index of deprivation to HCC1 indicating a significant positive relationship ($AIC=-24.76$, $p=0.005$). Season of the year during which the hair sample was obtained, which may have impacted upon the amount of time spent indoors or outdoors, had no significant impact on HNC1 or HCC1 (Kruskal-Wallis tests, HNC1 $p=0.32$, HCC1 $p=0.13$). There was a non-significant tendency for HCC1 to be higher in March-May compared to the other seasons, however this was confounded by the dogs seen at this time tending to be heavier. As several people lived in flats, only a subsection of owners had private gardens. However, whether the dogs in the non-smoking homes had access to such a space or not was not significantly associated with HNC1 (Wilcoxon rank-sum test, $p=0.61$). However, for HCC1 there was a non-significant decrease in those dogs with a private garden (Wilcoxon rank-sum test, $p=0.06$). In those who had private gardens, no significant difference was found in HNC1 or HCC1 if the owners used garden chemicals or not (Wilcoxon rank sum tests, HNC1 $p=0.52$, HCC1 $p=0.22$). The times per day the dog was walked or spent outdoors were not significantly

modelled to HNC1 (AIC=71.27, $p=0.18$; AIC=72.39, $p=0.37$, respectively). However, both the time being walked and the time spent outdoors were both significantly modelled to HCC1 (AIC=-28.53, $p<0.001$; AIC=-33.47, $p=0.01$). The longer the dog was taken on walks per day had a positive relationship with HCC1. This was time the dog spent away from the non-smoking home environment. Time spent outdoors was negatively related to HCC1. This included time spent in a private garden, and time spent outdoors was increased when access to a garden was available. Location was divided into inner city or large town, suburban and rural areas. There was no significant difference in HNC1 or HCC1 of dogs based on which on these types of locations they lived in (Kruskal-Wallis tests, HNC1 $p=0.08$, HCC1 $p=0.42$). However, it was noted that rural based dogs tended to have lower HNC1 on a plot of the data. If urban and suburban groups were combined, these dogs had higher HNC1 than rural based dogs (Wilcoxon rank-sum test, $p=0.03$). This comparison was not significant for HCC1 ($p=0.26$). While all the dogs examined in these analyses currently lived in non-smoking homes, some had been rehomed from owners who smoked and, in the case of 2 of the dogs, their owner stopped smoking cigarettes 3 years ago. Combining the dogs in the homes of former smokers and those adopted from smoking homes, and comparing these dogs with those who had been in non-smoking homes since leaving their breeder, there was a non-significant increase in HNC1 in those who had ever lived in smoking homes (Wilcoxon rank-sum test, $p=0.18$). The difference was less notable in HCC1 ($p=0.32$).

Following these analyses, it was considered why one dog in group 1 had a positive HNC1 based on ROC generated cut-off, and if there were reasons other than assay related error. This dog was a male and weighed 20.6kg. As previously mentioned the median weight in this group was 14.8kg so this dog was above this. It was a Staffordshire bull terrier, which have one of the highest cephalic indices of the breeds examined. It was 5 years old, one of the oldest dogs to take part. In addition, it lived in an inner city location. These factors combined, particularly its weight and location may have been contributed to the positive HNC1.

Variables related to HNC and HCC in ETS exposed dogs

As previously stated, HNC1 was not statistically significantly increased in group 3 dogs compared to group 2 dogs (Wilcoxon rank-sum test, $p=0.22$), nor was HCC1 increased in group 3 compared to group 2 ($p=0.58$). The non-significant difference in HNC1 may be due to the sample size as the groups visually appear different on the box and whisker plots. Therefore, analyses were conducted to explore whether any other variables could be associated with HNC1 and HCC1 other than smoking not occurring indoors which could be corrected for in analyses. Again, time point 1 measures were focussed on due to the higher number of measures available for analyses. Table A7-1 summarises the locations in which the dogs were exposed to ETS and results of tests to see if location where ETS exposure occurred was linked to HNC1 or HCC1. Regularity of exposure of any of the noted locations was not significantly related with HNC1 or HCC1.

Table A7-1 Locations where dogs are exposed to ETS
If exposure happens regularly (at least once a week), occasionally (less than weekly) or never. Kruskal-Wallis tests were conducted to explore whether the HNC1 or HCC1 were different in regularity of location exposure. KW p= Kruskal-Wallis test p value

	Regularly	Occasionally	Never	HNC1 KW p	HCC1 KW p
Close contact with owner	14	1	5	0.27	0.15
In pet's home	13	1	6	0.08	0.25
During car travel	2	5	13	0.50	0.61
In family or friends' home	2	7	11	0.85	0.91
Outdoors	19	1	0	0.43	0.31

The upper limit of the group of number of cigarettes smoked per day were totalled for the smokers within each dog's home. The total number of cigarettes were borderline significantly modelled to HNC1 (AIC=69.40, $p=0.048$), and highly significantly modelled to HCC1 (AIC=-0.20, $p<0.001$) (Figure A7.1A&B). Adjusting the number of cigarettes by the proportion of the day each person spent time with the dog generated similar results (HNC1: AIC=69.43; $p=0.049$; HCC1: AIC=0.16, $p<0.001$).

The linear model of dog age to HNC1 was not statistically significant (AIC=72.80, $p=0.76$). HCC1 non-significantly increased with dog age (AIC=3.74, $p=0.06$), but when number of cigarettes was included in the analysis, the relationship between HCC1 and dog age reduced in significance ($p=0.67$), as owners of older dogs tended to smoke more cigarettes per day. The linear model of dog weight to HNC1 was statistically significant (AIC=65.47, $p=0.007$), with dog weight being negatively related to HNC1. This relationship remained significant when number of cigarettes smoked per day was included (AIC=66.48, $p=0.009$). The negative relationship between dog weight and HNC1 was in contrast to that of the dogs in the non-smoking group, which displayed a positive relationship between HNC1 and dog weight (Figure A7.2). The linear model of dog weight to HCC1 was borderline significant (AIC=3.22, $p=0.049$). This relationship was no longer significant when number of cigarettes per day in the home was included ($p=0.42$ for the weight variable). Similarly to dog weight, breed height was negatively related to HNC1 (AIC=67.63, $p=0.02$). This relationship remained significant when number of cigarettes smoked per day was included in the analysis (AIC=67.04, $p=0.01$). HCC1 also decreased with breed height (AIC=0.28, $p=0.01$), but the relationship was no longer significant when number of cigarettes smoked per day was included. The linear model of breed cephalic index to HNC1 was not statistically significant (AIC=53.96, $p=0.19$). HCC1 was positively related to breed cephalic index (AIC=3.18, $p<0.001$), but the relationship was no longer significant when number of cigarettes smoked per day was included in the analysis ($p=0.38$). Regarding coat-related factors, Kruskal-Wallis tests indicated that there were significant differences in HNC1 and HCC1 between fur lengths ($p=0.04$, $p=0.02$, respectively).

Pairwise Wilcoxon rank sum tests with Holm p value correction did not reveal any pairwise differences in HNC1 between lengths, but a significant difference in HCC1 was found between dogs with short and dogs with long fur ($p=0.04$). However, this difference was no longer significant when number of cigarettes per day was included in a linear model of fur length to HCC1. Fur type, single or double coat, fur colour and shedding rate were not significantly related to HNC1 or HCC1 ($p>0.1$ for all). In addition, no significant differences in HNC1 or HCC1 were found with brushing or bathing frequency ($p>0.1$ for all).

Of the 6 dogs in smoking homes with negative cotinine values based on the cut-offs generated from the ROC analysis, 3 were in group 2 so were protected from some exposure within the home. There were no obvious reasons why the other dogs had negative HCC1 values (aside from assay error), and did not have any shared characteristics of note.

Gender

Studies in humans have shown an increased rate of nicotine metabolism in women (e.g. Benowitz et al. 2006). Therefore, dog gender was investigated for associations with HNC1 and HCC1. Given the small number of females, and that they were spread across the exposure groups, gender was examined as a variable with all exposures combined. There was no significant difference in HNC1 with gender (Wilcoxon rank-sum test, $p=0.97$). HCC1 was increased in female dogs, but not significantly ($p=0.23$). The ratio of HNC1:HCC1 was lower in female dogs than male dogs. The test did not reach significance ($p=0.06$), but a trend can be seen in Figure A7.3. Gender was a significant explanatory variable when included in a linear model of number of cigarettes per day to HNC1:HCC1 ratio, and the model was highly significant ($AIC=156.41$, $p<0.001$).

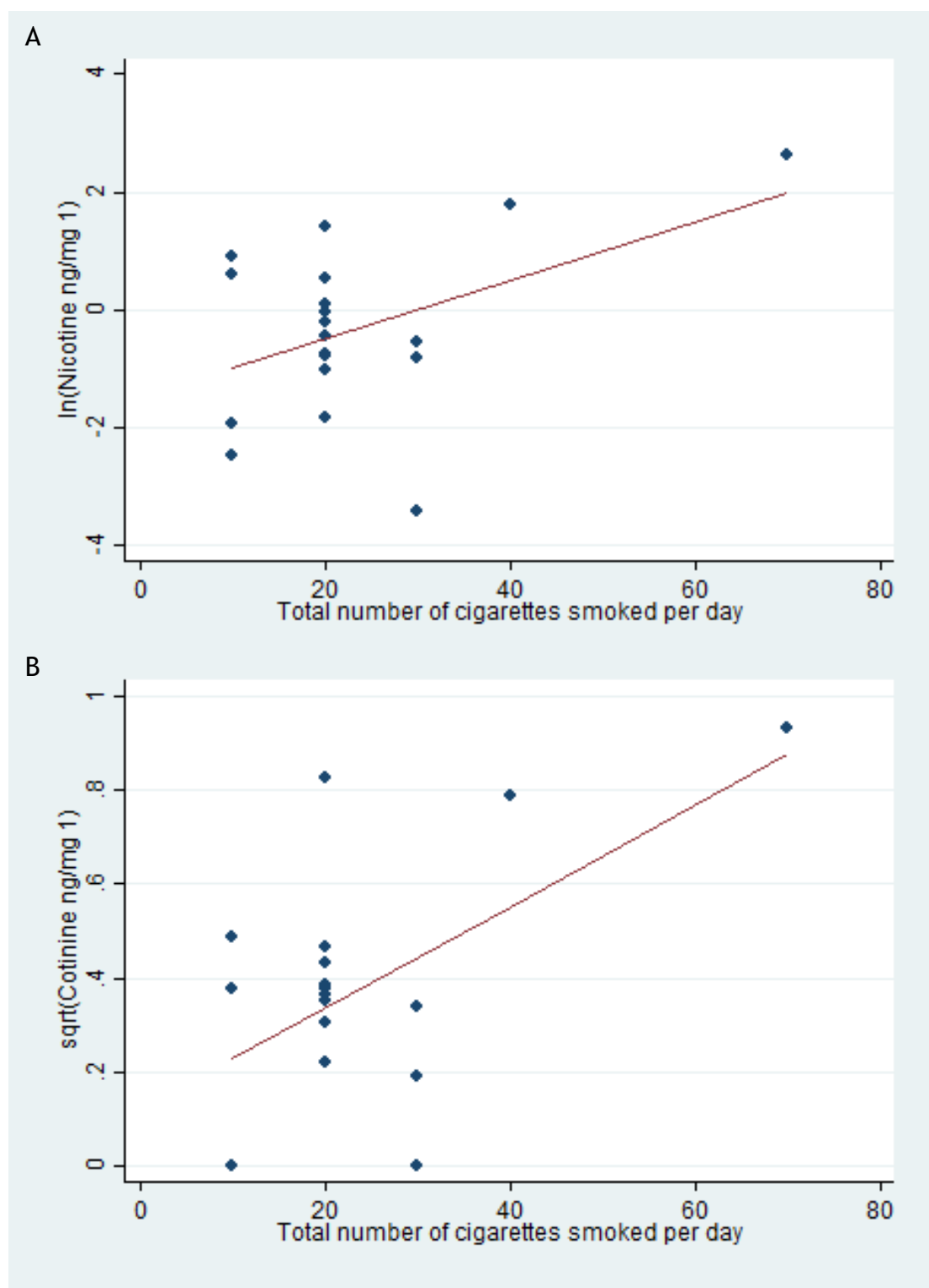


Figure A7.1 Upper limit of number of cigarettes smoked in dog's home by HNC1 (A) and HCC1 (B)
 Number of cigarettes smoked in the home was significantly related to HNC1 (AIC=69.40, $p=0.048$) and HCC1 (AIC=-0.20, $p<0.001$) (n=19, 17 male 2 female)

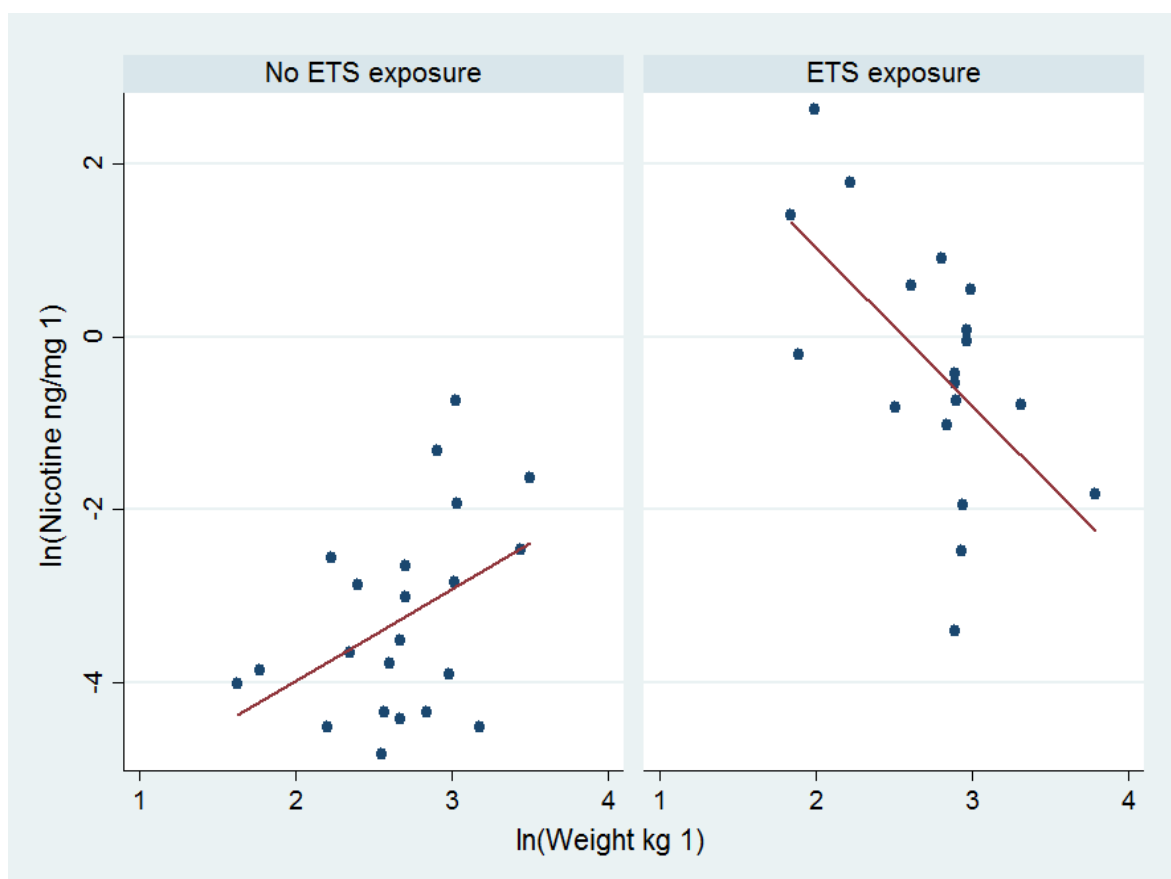


Figure A7.2 HNC1 and dog weight
By whether the dog was in a non-smoking or a smoking home (n=41, 22 no ETS exposure, 19 ETS exposure)

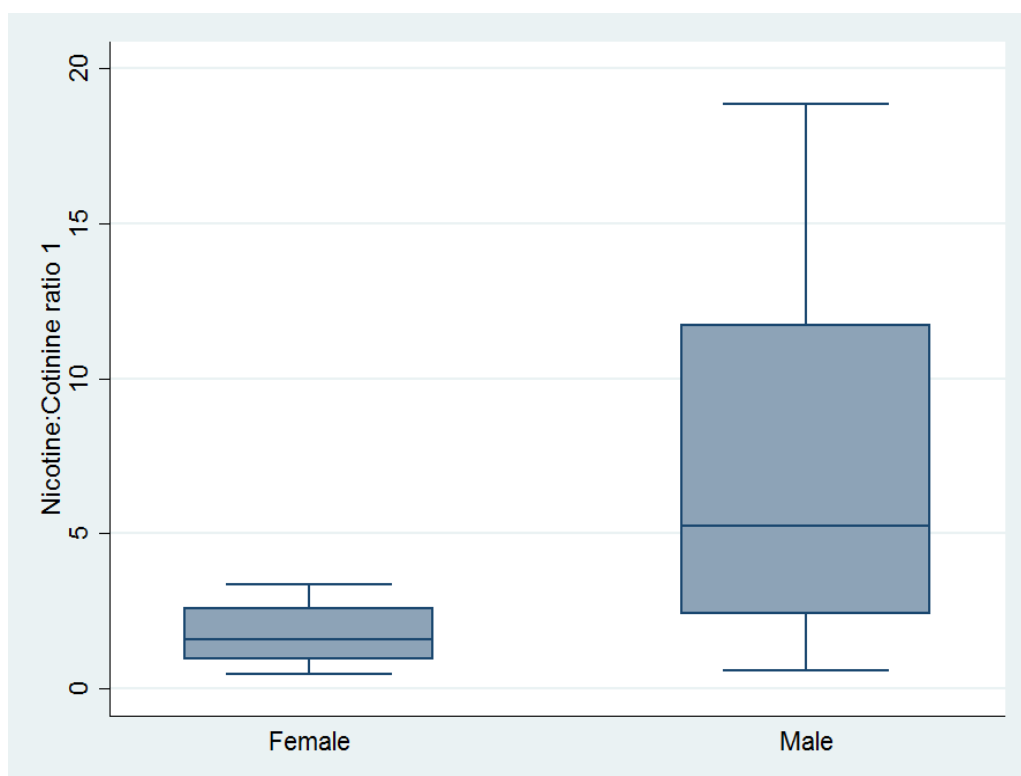


Figure A7.3 The ratio of nicotine to cotinine at time point 1 by sex (n=41, 37 male, 4 female)

Appendix 8: Chapter 4 additional analyses

The following analyses were conducted to determine if the causes of inter-individual variability in LTL in the qPCR optimisation samples. Neither weight nor height were significantly associated with RTL ($F=0.34_{(1,79)}$, $R^2=0.004$, $p=0.56$, and $F=0.81_{(1,79)}$, $R^2=0.01$, $p=0.37$, respectively) (Figures A8.1, A8.2). If age was added as an explanatory variable, both weight and height were significantly associated with RTL ($F=5.22_{(2,78)}$, $R^2=0.12$, $p=0.007$ and $F=5.73_{(2,78)}$, $R^2=0.12$, $p=0.007$, respectively). Breed average cephalic index (CI) was also not significantly associated with RTL ($F=3.15_{(1,72)}$, $R^2=0.04$, $p=0.08$, Figure A8.3), but was when age was added as an explanatory factor ($F=4.79_{(2,70)}$, $R^2=0.12$, $p=0.01$). The breed estimated effective population size (EEPS), a measure of inbreeding, was not significantly associated with RTL ($F=2.35_{(1,70)}$, $R^2=0.03$, $p=0.13$, Figure A8.4), but again when age was added as an explanatory factor the linear model was statistically significant ($F=6.41_{(2,68)}$, $R^2=0.16$, $p=0.003$). When age, cephalic index and estimated effective population size were used as explanatory variables, the linear regression to RTL was significant ($F=6.36_{(3,60)}$, $R^2=0.24$, $p<0.001$).

A student's t-test showed there was no significant difference in RTL between neutered and entire dogs (Figure A8.5, $p=0.32$), despite a Wilcoxon-rank sum test demonstrating that dogs in the neutered group were significantly older ($p=0.01$). The difference in RTL between neutered and entire dogs was still not statistically significant when age was corrected for.

While the aim was to avoid individuals with a cancer diagnosis, 7 dogs were subsequently discovered to have a diagnosed malignancy. There was a statistically significant difference in RTL between dogs diagnosed and not diagnosed with a malignancy (t-test, $p=0.04$, Figure A8.6). When age and diagnosis were used as explanatory variables, the model to RTL was statistically significant ($F=5.95_{(3,77)}$, $R^2=0.19$, $p=0.001$). When EEPS and CI were also included as explanatory variables, the model was improved ($F=7.77_{(4,59)}$, $R^2=0.35$, $p<0.001$).

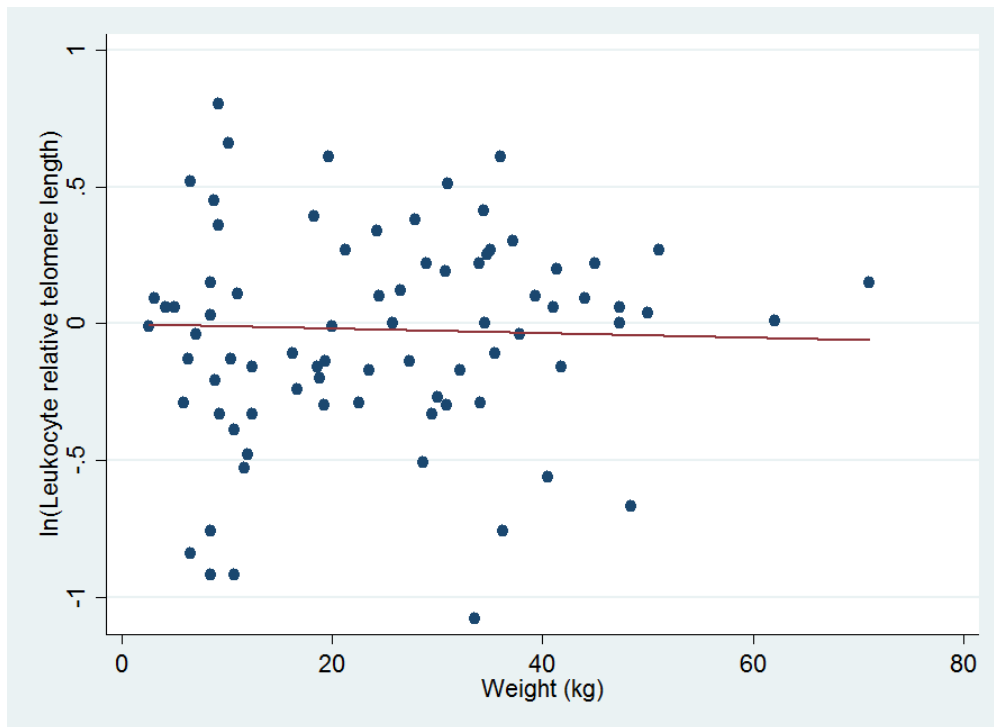


Figure A8.1 Leukocyte telomere length by dog weight
 Scatter plot of natural log of relative telomere length in leukocytes in dogs by weight of the dog in kilograms, with regression line. Linear regression was not statistically significant ($R^2=0.004$, $p=0.56$)

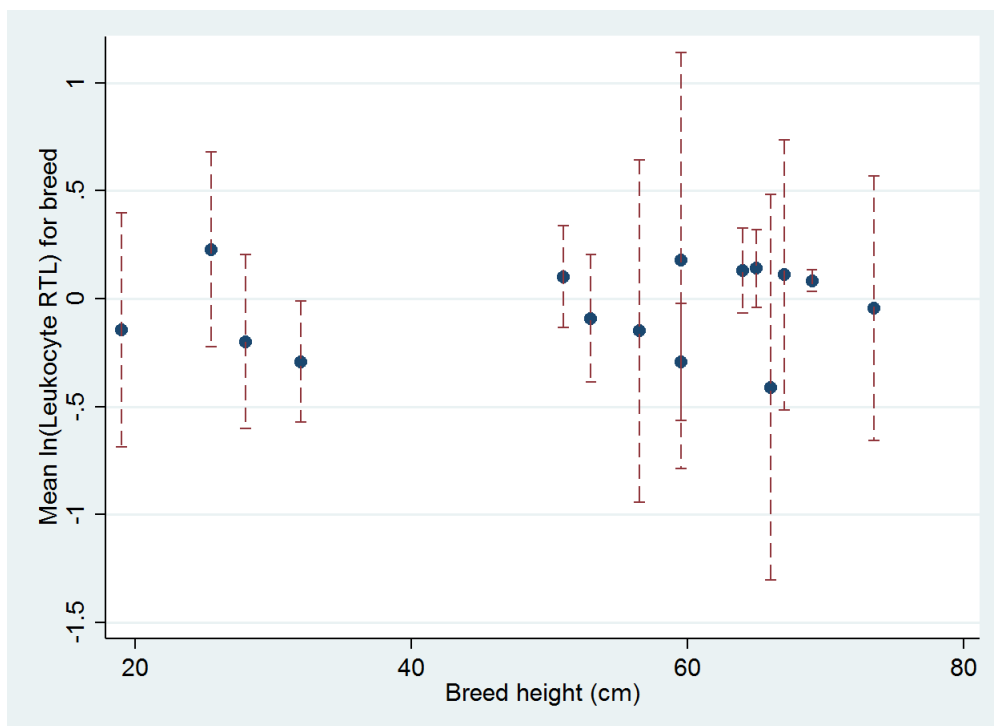


Figure A8.2 Leukocyte telomere length by breed height
 Scatter plot of mean natural log of relative telomere length in leukocytes in dogs by the average height for the breed, with 95% confidence intervals (dashed lines). The linear model was not statistically significant ($R^2=0.01$, $p=0.37$)

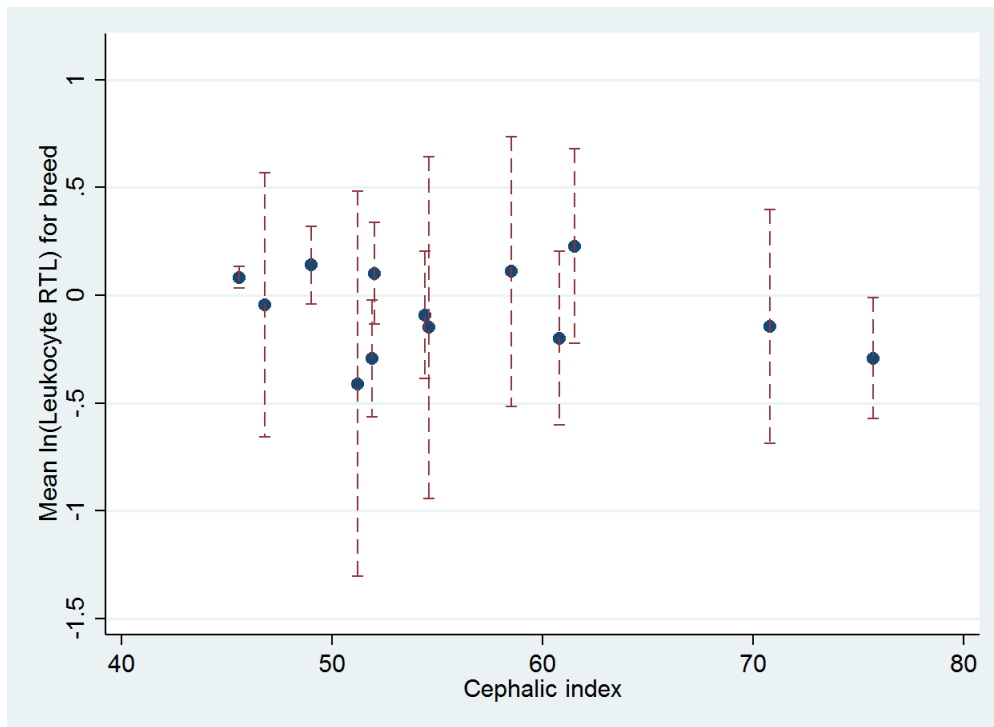


Figure A8.3 Leukocyte telomere length by breed cephalic index

Scatter plot of mean natural log of relative telomere length in leukocytes by breed cephalic index, with 95% confidence intervals (dashed lines). The linear model was not statistically significant ($R^2=0.04$, $p=0.08$).

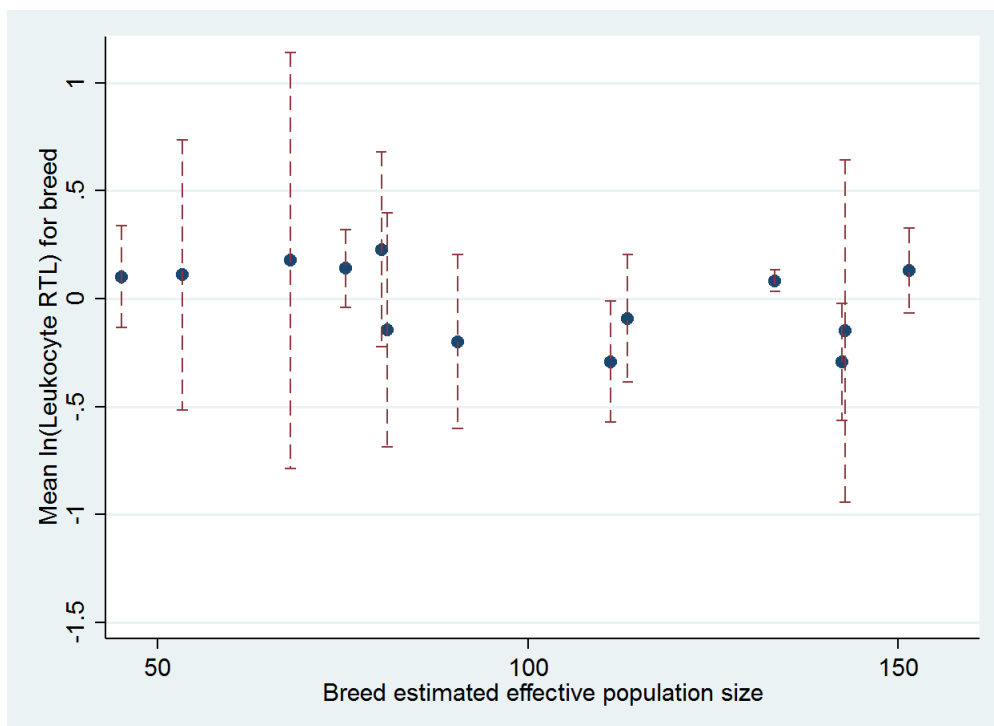


Figure A8.4 Leukocyte telomere length by breed EEPs

Scatterplot of mean natural log of relative telomere length in leukocytes in dogs the estimated effective population size of the breed, with 95% confidence intervals (dashed lines). The linear model was not statistically significant ($R^2=0.03$, $p=0.13$).

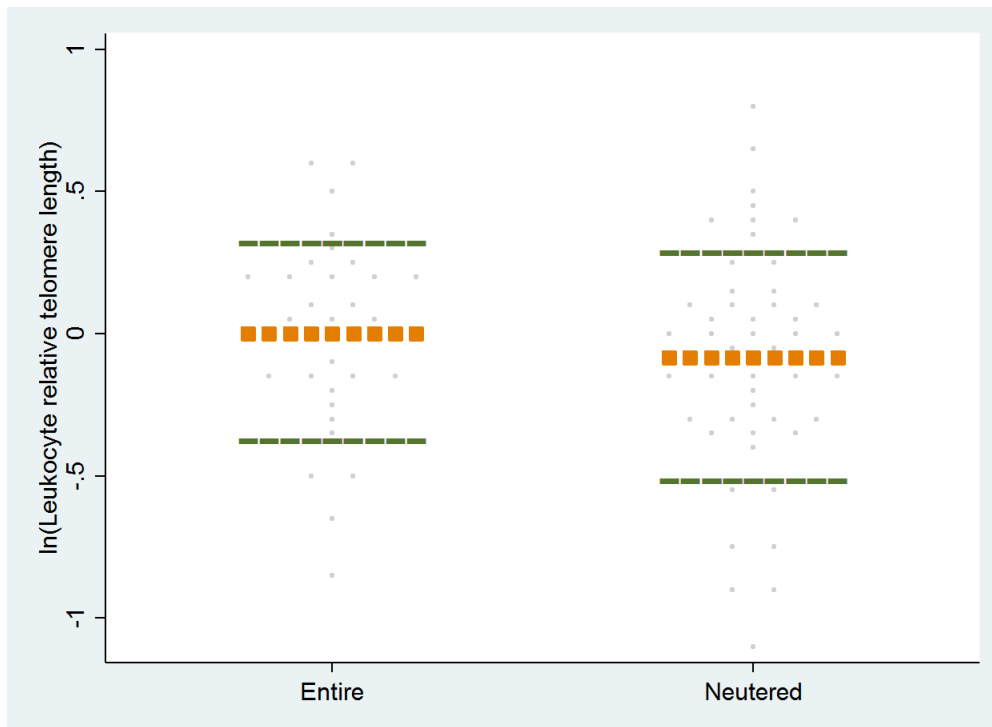


Figure A8.5 Leukocyte telomere length by neuter status

Dot plot of relative LTL by whether the dog was entire or neutered. Mean value of LTL for each is displayed in orange, mean \pm standard deviation in green, with the individual LTL values in grey. There was no significant difference in RTL between the groups ($p=0.32$)



Figure A8.6 Leukocyte telomere length by whether the dog had a malignancy diagnosed

Dot plot of relative LTL by whether the dog was diagnosed with a malignancy. Mean value of LTL for each is displayed in orange, mean \pm standard deviation in green, with the individual RTL values in grey. There was a significant difference between the groups ($p=0.04$).

Biochemical and haematological measures

Biochemistry data were available for all except three dogs and haematological data for all bar two dogs. However, glucose concentration was measured in only 71 dogs and phosphate concentration in 62 dogs. In order to look for confounding factors prior to exploring associations between RTL and biochemical and haematological measures, each measure was assessed against age, weight and breed life expectancy using Spearman's rank correlation. The Spearman's rho value is displayed for each correlation test and the values in bold indicate that the correlation was statistically significant (Table A8-1). The neutrophil to lymphocyte ratio and lymphocyte to monocyte ratio were examined in addition to the standard haematology measures.

While 8 measures were significantly correlated with age, only alkaline phosphatase concentration (ALKP) ($F=4.23_{(1,76)}$, $R^2=0.05$, $p=0.04$), glucose concentration ($F=6.08_{(1,67)}$, $R^2=0.08$, $p=0.02$) and neutrophil to lymphocyte ratio ($F=13.04_{(1,77)}$, $R^2=0.14$, $p<0.001$) were significantly associated with RTL. None of the renal parameters or electrolytes, the other liver and protein parameters, red cell parameters, or platelet parameters were significantly associated with RTL. Concentrations of cholesterol and triglyceride were not significantly associated with RTL. White cell parameters, except neutrophil to lymphocyte ratio, were also not significantly associated with RTL. However, when factors such as age and EEPS were added as explanatory variables, several of the biochemical and haematological parameters were significantly associated with RTL. After inclusion of age alone, potassium concentration was significantly associated with RTL ($F=4.99_{(2,75)}$, $R^2=0.12$, $p=0.009$) as was chloride concentration ($F=4.97_{(2,75)}$, $R^2=0.12$, $p=0.009$), creatinine concentration ($F=5.10_{(2,75)}$, $R^2=0.12$, $p=0.008$) and mean platelet volume (MPV) ($F=5.08_{(2,76)}$, $R^2=0.12$, $p=0.008$). Inclusion of EEPS as an explanatory variable resulted in lymphocyte to monocyte ratio being significantly associated with RTL ($F=4.17_{(2,67)}$, $R^2=0.11$, $p=0.02$). When age and EEPS were added as explanatory factors, sodium concentration was significantly associated with RTL ($F=4.45_{(3,65)}$, $R^2=0.17$, $p=0.007$), as was calcium concentration ($F=4.87_{(3,65)}$, $R^2=0.18$, $p=0.004$) as well as globulin concentration ($F=4.44_{(3,65)}$, $R^2=0.17$,

$p=0.007$), red cell count (RBC) ($F=5.03_{(3, 66)}$, $R^2=0.19$, $p=0.003$), haemoglobin concentration ($F=4.46_{(3, 66)}$, $R^2=0.17$, $p=0.006$), haematocrit percentage (HCT) ($F=4.25_{(3, 66)}$, $R^2=0.16$, $p=0.008$), mean corpuscular haemoglobin concentration (MCHC) ($F=4.40_{(3, 66)}$, $R^2=0.17$, $p=0.007$) and red cell distribution width percentage (RDW) ($F=4.19_{(3, 66)}$, $R^2=0.16$, $p=0.009$).

Figure A8.7 illustrates the correlation between age, weight, life expectancy and RTL with serum phosphate concentration. Phosphate concentration was significantly negatively correlated with age, but unrelated to RTL. Median serum phosphate concentration was not significantly correlated with breed life expectancy, but there was a downward trend and the breed with the shortest life expectancy, the Dogue de Bordeaux, had the highest median serum phosphate (2.2mmol/L). In addition, breed life expectancy was not correlated with breed mean RTL (Spearman's $\rho=-0.30$, $p=0.28$), but was significantly negatively correlated with median breed weight (Spearman's $\rho=-0.64$, $p=0.01$), Figure A8.8. Dogs that were known to have died during the episode of treatment in which the blood sample used for analysis was taken were grouped and compared to the other dogs who were not recorded to have died during the episode. It was not known how much longer the dogs in the alive group lived, some may have died soon after. Those in the "died" group included those euthanised, as there were only 7 dogs known to have died in total. Illnesses related to deaths, or reasons for euthanasia, were hepatopathy, granulomatous meningoencephalitis, degenerative cerebral disease, spinal cord embolism, renal disease and two cases of osteosarcoma. A t-test showed there was not a significant difference in RTL between those who died and did not ($p=0.12$), but when age and death were used as explanatory variables in linear regression to RTL, the model was significant ($F=4.07_{(3,77)}$, $R^2=0.14$, $p=0.01$).

Table A8-1 Correlations between biochemical and haematological blood measures and age, weight and breed life expectancy

Spearman's rho correlation values are shown in the cells. Rho values in bold indicate the correlation was statistically significant, with *= $p<0.05$, **= $p<0.01$, *= $p<0.001$.**

	Age	Weight	Breed life expectancy
Sodium	-0.04	0.08	-0.05
Potassium	-0.13	0.03	-0.22*
Chloride	-0.04	0.21	0.04
Calcium	-0.06	-0.08	0.09
Phosphate	-0.29*	-0.23	0.02
Urea	0.23*	0.31**	-0.13
Creatinine	0.23*	0.56***	-0.16
Glucose	0.04	-0.19	0.14
Cholesterol	0.18	0.02	-0.13
Triglyceride	0.26*	-0.06	-0.003
Alkaline phosphatase	0.29**	-0.22*	0.23*
AST	0.14	0.11	-0.05
ALT	0.06	0.03	-0.06
GGT	0.06	0.21	0.17
Total protein	0.06	-0.05	0.02
Albumin	-0.08	-0.17	0.10
Globulin	0.11	0.05	-0.07
Albumin:globulin ratio	-0.18	-0.10	0.05
RBC	0.04	0.27*	-0.07
Haemoglobin	0.01	0.29**	-0.07
HCT	-0.01	0.26*	-0.04
MCV	-0.20	-0.20	0.13
MCH	-0.19	-0.05	-0.06
MCHC	0.12	0.27*	-0.20
RDW	0.05	-0.19	0.05
Leukocytes	0.01	-0.10	-0.006
Neutrophils	0.08	-0.11	0.02

Lymphocytes	-0.37***	-0.04	-0.04
Neutrophil:lymphocyte ratio	0.27*	-0.07	0.08
Monocytes	0.09	-0.15	0.04
Lymphocyte:monocyte ratio	-0.28*	0.11	-0.09
Eosinophils	-0.15	0.11	-0.09
Basophils	-0.14	-0.12	0.26*
Platelets	0.12	-0.29**	0.10
MPV	-0.12	0.22*	-0.28*
PCT	0.11	-0.27*	0.02
PDW	-0.11	0.25*	-0.29**

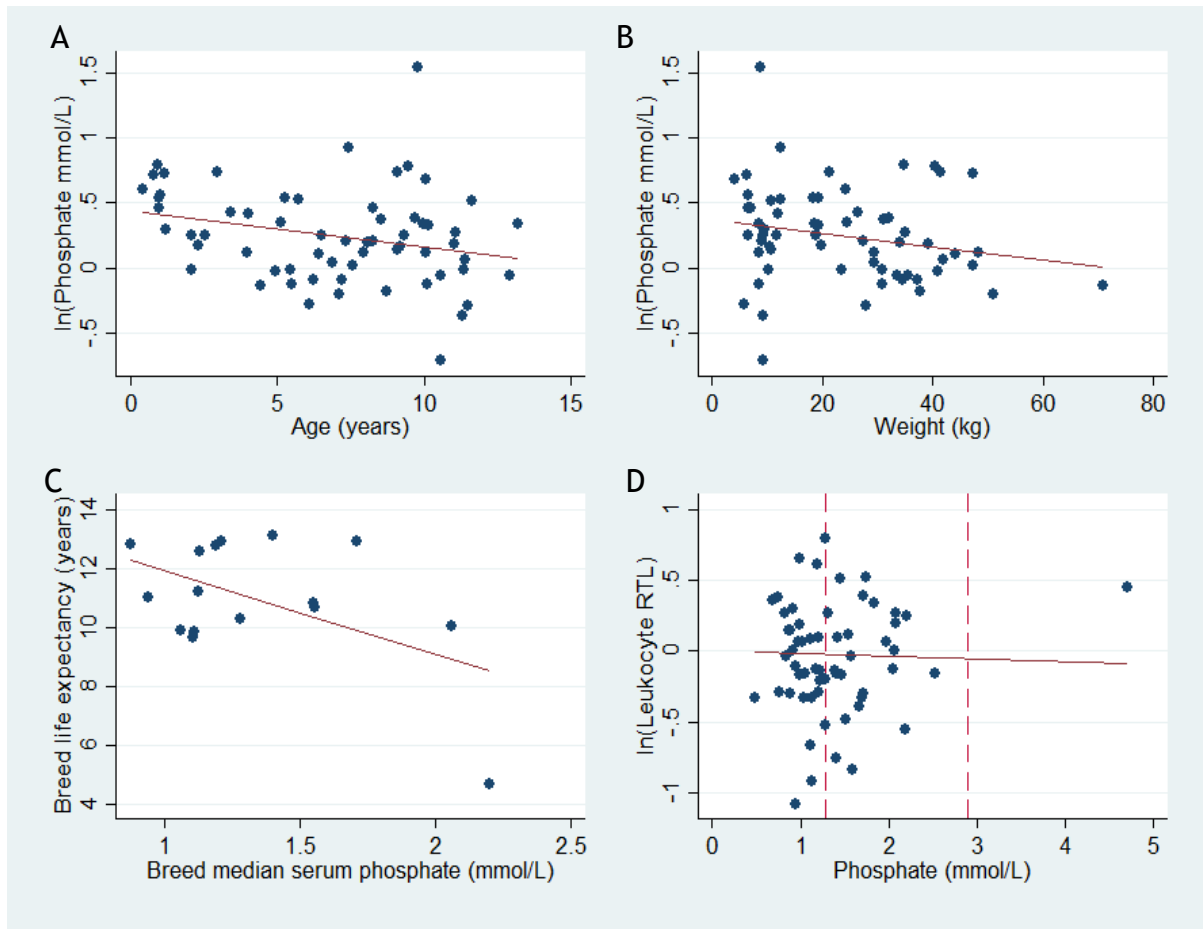


Figure A8.7 Serum phosphate concentration

Scatterplots of log phosphate by dog age (A), log phosphate by dog weight (B), median serum phosphate by breed life expectancy (C) and log telomere length by serum phosphate (D), with regression line. The red dashed lines in plot D represent the reference range values given by the diagnostics laboratory. There was a significant linear regression between log phosphate and age ($R^2=0.07$, $p=0.04$), but no significant regression between log phosphate and weight ($R^2=0.04$, $p=0.11$), log telomere length and phosphate ($R^2=0.01$, $p=0.53$) or breed life expectancy and median serum phosphate (Spearman's $\rho=-0.04$, $p=0.90$).

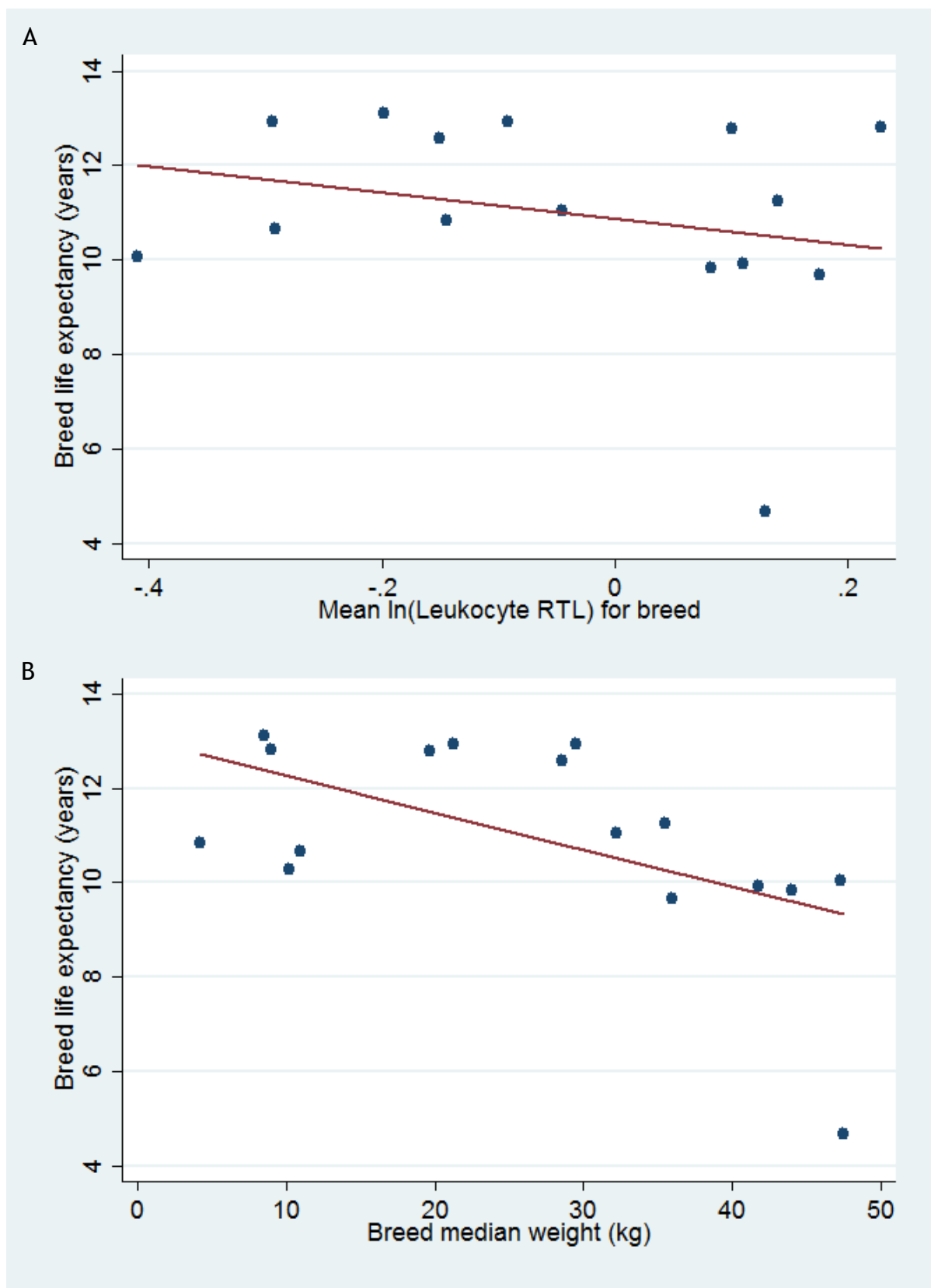


Figure A8.8 Breed life expectancy by leukocyte telomere length and by dog weight
 Scatterplots of breed life expectancy by breed mean relative telomere length (A) and breed median weight (B), with regression line. There was no significant correlation between life expectancy and telomere length (Spearman's $\rho = -0.30$, $p = 0.28$), but there was between life expectancy and weight (Spearman's $\rho = -0.64$, $p = 0.01$).

Other factors

Index of deprivation

The index of deprivation was obtained for each owner using their postcode. Five owners lived in England and therefore were not included, as the measure was obtained from the Scottish government website. The linear model of the index of deprivation to RTL was not significant (Figure 4.24, $F=3.22_{(1,74)}$, $R^2=0.04$, $p=0.08$). Index of deprivation was still not a statistically significant explanatory variable when other factors such as age, cephalic index and estimated effective population size were included.

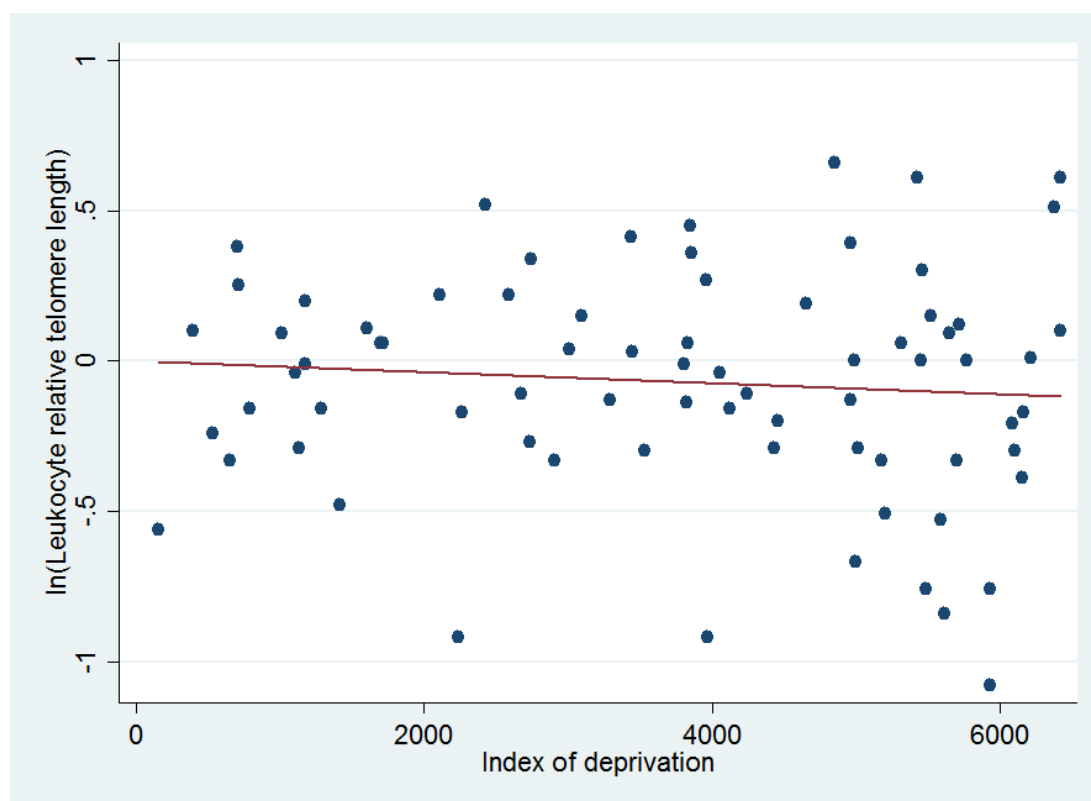


Figure A8.9 Leukocyte telomere length in dogs by owner index of deprivation
Scatter plot of natural log of relative telomere length in leukocytes by owner index of deprivation, with regression line. The linear model was not significant ($R^2=0.04$, $p=0.08$).

Extraction kit

A portion of the bloods were DNA extracted using a Qiagen kit and the remaining using a Macherey Nagel kit, as described in the methods section. A t-test showed that the log RTLs were significantly longer when the samples were extracted using a Qiagen kit (Figure A8.9, $p=0.01$). However, when age in the two groups was compared using Wilcoxon rank sum tests, the Macherey Nagel extracted group were significantly older ($p=0.01$) and had higher neutrophil to lymphocyte ratios ($p=0.03$).

The 260/280 ratios of samples extracted using the Qiagen kit were significantly higher than those extracted using the Macherey Nagel kit (Wilcoxon rank sum test, $p=0.003$). The converse was true for the 260/230 ratio; those extracted with the Macherey Nagel kit had significantly higher ratios (Wilcoxon rank sum test, $p<0.001$). Linear models revealed no significant relationship between RTL and 260/280 ($p=0.35$) or 260/230 ($p=0.84$) ratio. There was, however, a statistically significant negative correlation between 260/280 ratio and telomere C_T (Spearman's $\rho=-0.27$, $p=0.01$), but no significant correlation between telomere C_T and 260/230 ratio (Spearman's $\rho=0.06$, $p=0.64$). There were no statistically significant correlations between HBD C_T and 260/280 ratio (Spearman's $\rho=-0.008$, $p=0.94$), or HBD C_T and 260/230 ratio (Spearman's $\rho=-0.06$, $p=0.63$). In addition, there was a significant negative correlation between 260/280 and age (Spearman's $\rho=-0.27$, $p=0.02$) and a statistically significant positive correlation between 260/230 ratio and age (Spearman's $\rho=0.25$, $p=0.04$). Table A8-2 shows the correlation values for white cell counts and ratios against DNA quality measures and C_T values from the telomere and HBD PCRs. The white cell count of the sample was significantly positively correlated with 260/230 ratio, as well as being negatively correlated with C_T values in both PCRs.

Table A8-2 Correlations between white cell differentials, DNA quality and qPCR crossing points

Correlations between white cell differential values, DNA sample absorbance ratios and crossing points in qPCR assays. The values displayed are the correlation coefficients. *=p<0.05, **=p<0.01, *=p<0.001**

	260/280	260/230	Ct Tel	Ct HBD
White cell count	0.20	0.39***	-0.29**	-0.26*
Neutrophil count	0.18	0.43***	-0.14	-0.25*
Lymphocyte count	0.10	-0.14	0.07	-0.09
Neutrophil:lymphocyte ratio	0.05	0.29*	-0.18	-0.18
Monocyte count	0.26*	0.30*	-0.08	-0.19
Lymphocyte:monocyte ratio	-0.18	-0.33**	0.24*	0.14
Eosinophil count	0.09	-0.01	0.13	-0.04
Basophil count	-0.07	-0.03	0.05	-0.09

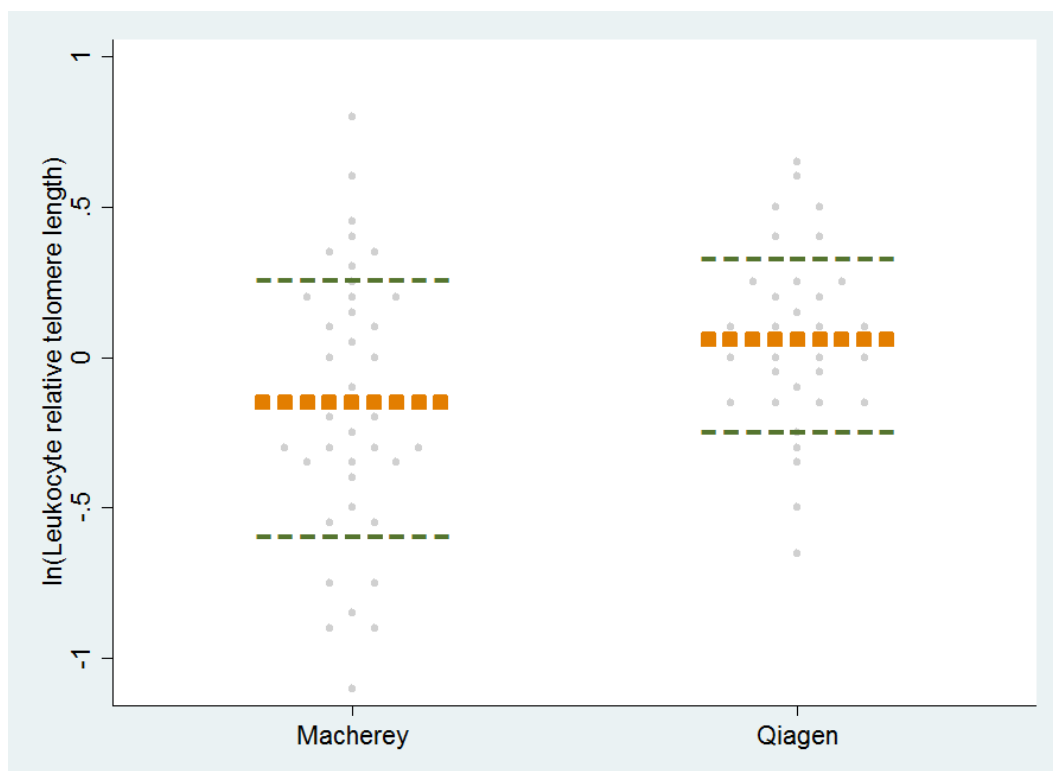


Figure A8.10 Leukocyte telomere length by the kit used to extract DNA

Dot plot of natural log of relative telomere length by whether the DNA had been extracted using a kit by Macherey Nagel or Qiagen. The mean value is shown in orange and 95% confidence intervals in green. A student's t-test was performed which showed a significant difference in log of LTL between the groups ($p=0.01$)

Overall linear regression model

To work out the combination of explanatory variables from all those examined for the qPCR optimisation samples that would best explain inter-individual variation in RTL, the “drop1” function was used. As there were so many variables, there were not enough degrees of freedom in the model to permit all explanatory variables to be tested in one attempt. Therefore, one model, “model1”, was developed with breed and without phosphate and glucose concentrations, as not all dogs had these variables tested in biochemical analyses and breed took up many degrees of freedom. In addition, this model did not include CI, EEPS, breed lifespan or breed height as these were the same for members of each breed (Table A8-3). A second model, “model2” was developed without breed as a variable, but with CI, EEPS, lifespan and height included as starting variables (Table A8-4). The drop1 combined with an “F” test provides p values for each variable. The highest p value variable is removed and then the model re-tested, until all variables have a p value of less than 0.05. As the intercept was not significant for model1, the model was fitted without an intercept. Interestingly, age was not retained as an explanatory variable in model1.

Table A8-3 model1, generated using drop1 to decide upon explanatory variables to model RTL

Parameter	Estimate	Standard error	t value	Pr (> t)
Breed- Akita	-1.35	0.77	-1.74	0.09
Breed- Bichon frise	-0.90	0.78	-1.15	0.26
Breed- Border collie	-1.38	0.77	-1.78	0.08
Breed- Cavalier King Charles spaniel	-1.88	0.80	-2.34	0.02
Breed- Chihuahua	-1.42	0.76	-1.88	0.07
Breed- Dalmatian	-1.40	0.74	-1.89	0.07
Breed- Dobermann	-1.27	0.77	-1.65	0.11
Breed- Dogue de Bordeaux	-1.04	0.76	-1.37	0.18
Breed- English springer spaniel	-1.18	0.75	-1.57	0.12
Breed- Flat coated retriever	-1.13	0.77	-1.47	0.15
Breed- Greyhound	-0.99	0.77	-1.29	0.20
Breed- Rhodesian ridgeback	-1.87	0.79	-2.37	0.02
Breed- Siberian husky	-1.29	0.68	-1.88	0.07
Breed- Weimaraner	-0.96	0.74	-1.29	0.20
Breed- West Highland white terrier	-1.28	0.76	-1.70	0.10
Diagnosis- Cancer	-0.53	0.13	-4.22	0.0001
Sodium:potassium ratio	-0.02	0.009	-2.34	0.02
Cholesterol	-0.03	0.01	-2.68	0.01
Albumin	0.04	0.01	2.65	0.01
Albumin:globulin ratio	-0.79	0.31	-2.54	0.01
Red blood cell count	-0.09	0.04	-2.34	0.02
Lymphocyte count	0.14	0.06	2.55	0.01
Monocyte count	-0.22	0.08	-2.60	0.01
Platelet count	0.003	0.001	2.06	0.04
Mean platelet volume	0.06	0.02	2.86	0.006
Plateletcrit	-2.37	1.11	-2.14	0.04
Platelet distribution width	0.03	0.008	3.28	0.002

Multiple R ²	0.69			
Adjusted R ²	0.51			
F (27,48)	3.92			
p-value	1.819x10 ⁻⁵			

Table A8-4 model2, generated using drop1 but excluding breed as an explanatory variable

Parameter	Estimate	Standard error	t value	Pr (> t)
Intercept	-5.22	1.72	-3.03	0.004
Age	-0.03	0.01	-2.53	0.02
Diagnosis- Cancer	-0.39	0.14	-2.75	0.008
Extraction kit- Qiagen	0.20	0.07	2.81	0.007
Estimated effective population size	-0.004	0.001	-3.83	0.0004
Cephalic index	-0.01	0.004	-3.49	0.001
Potassium	0.78	0.18	4.36	0.00007
Sodium:potassium ratio	0.10	0.02	4.15	0.0001
Mean cell volume	-0.02	0.007	-2.20	0.03
White cell count	-0.08	0.04	-2.10	0.04
Neutrophil count	0.11	0.04	2.40	0.02
Neutrophil:lymphocyte ratio	-0.01	0.004	-3.03	0.004
Platelet distribution width	0.01	0.005	2.15	0.04
Multiple R ²	0.66			
Adjusted R ²	0.58			
F (12,47)	7.70			
p-value	1.23x10 ⁻⁷			

Appendix 9: Studies examining telomere length and smoking

Studies examining relationship between smoking and telomere length in humans

Community and occupational based recruitment studies

Abbreviations

CS/Lo	Cross sectional/longitudinal telomere length measurement
M	Male
F	Female
#	Number of study participants
%Smoker	Percentage of study participants classed as active smokers
PC	Phenol/chloroform
IP	Isopropanol precipitation
CL	Cell lysis
SO	Salting-out
SI	Sodium iodide
MB	Magnetic bead
TRF	TRF analysis
%COV	Percentage inter-assay coefficient of variation
Cat	Categorical
Cont	Continuous
-ve	Negative (active smoking status or increasing pack years decreased telomere length)
+ve	Positive (active smoking status or increasing pack years increased telomere length)
DM	Diabetes mellitus

MDD	Major depressive disorder
BPD	Bipolar disorder
SLE	Systemic lupus erythematosus
TAA	Thoracic aortic aneurysms
AAA	Abdominal aortic aneurysm
COPD	Chronic obstructive pulmonary disease
CCU	Coronary care unit
GI	Gastrointestinal
HIV	Human immunodeficiency virus
CABG	Coronary artery bypass graft

	Study group	CS/Lo	# Sex	%Smoker	Age	Healthy?	Tissue	DNA Extraction	Measure COV%	Relation with age	Smoking measure	Effect
Barbieri et al. 2009	Residents in the Campania region	CS	476 MF	<25	45-59	Yes	Blood	NS	TRF NS	Yes	Cat	No
Boccardi et al. 2013	Residents in the Campania region	CS	217 MF	<25	60+	Yes	Blood	Column	qPCR 1.98	Yes	Cat	Yes -ve
Borghini et al. 2015	Endurance athletes plus controls	CS	62 MF	<25	45-59	Yes	Saliva	Column	qPCR 4.6-7	No	Cat	No
Carty et al. 2015	Enrolled by the Women's Health Initiative	CS	1525 F	<25	60+	Yes	Blood	Unclear	TRF 2	Yes	Cat	Yes, -ve
Chan et al. 2010	Invited to health check	CS	2006 MF	<25	60+	Yes	Blood	PC	qPCR 11.2/14.2	No	Cat	No
Cui et al. 2014	Community based cohort populations	CS	4351 MF	25-49	45-59	CHD, controls	Blood	Auto	qPCR 1.28	Yes	Cat	No

Hoxha et al. 2009	Traffic officers and office workers	CS	134 MF	50+	NS	Yes	Blood	PC	qPCR NS	Yes	Both	Yes -ve
Kahl et al. 2016	Community sample	CS	124 MF	<25	<45	Yes	Blood	Column	qPCR NS	No	Cat	Yes -ve
Kasielski et al. 2015	Routine physical examination attendees	CS	28 MF	50+	<45	Yes	Blood	Column	qPCR NS	NS	Cat	No
Kiefer et al. 2008	Pre- and post-menopausal women	CS	36 F	<25	<45	Yes	Blood	NS	qPCR NS	No	Cat	No
Laine et al. 2015	Elite athletes & controls	CS	586 M	<25	60+	Yes	Blood	NS	qPCR 7.14	Yes	Cat	No
Latifovic et al. 2016	Healthy volunteers	CS	477 MF	<25	NS	Yes	Blood	IP	qPCR 6.39	Yes	Both	Yes -ve
Li et al. 2011	Rubber industry workers	CS	157 MF	25-49	<45	Yes	Blood	Column	qPCR 6.4	Yes	Cat	No
Li et al.	Healthy	CS	364	50+	<45	Yes	Blood	CL	qPCR	Yes	Cat	Yes -ve

2013	volunteers		M						7.2			
Lynch et al. 2016	Recruits from Centers for Population Health and Health Disparities	CS	1510 MF	50+	45-59	15.7% cancer	Blood	Mix	TRF 1.25	No	Cat	No
Pawlas et al. 2015	Lead smelters & controls	CS	394 M	25-49	<45	Yes	Blood	Column	qPCR NS	Yes	Cat	No
Raymond et al. 2014	Families recruited	CS	580 MF	<25	<45	Yes	Blood	SO	qPCR NS	Yes	Cat	No
Satoh et al. 1996	Company employees & former poison gas factory workers	CS	352 MF	NS	NS	Yes	Blood	SI	TRF NS	Yes	Cat	No
Shin et al. 2010	Community based health survey	CS	84 MF	25-49	45-59	Yes	Blood	SO	qPCR 7.5	No	Cat	No
Song et al.	Healthy	CS	103	NS	NS	Yes	Blood	SO	qPCR	Yes	Cont	Yes -ve

2010	volunteers		NS						NS			
Tyrka et al. 2015	Community sample	CS	335 MF	<25	<45	Mental health issues	Blood	NS	qPCR NS	No	Cat	Yes -ve
Walters et al. 2014	General population recruits	CS	58 MF	50+	<45	Yes	Blood & Small airway epithelium	SO	TRF NS	Only in non-smokers	Both	Yes -ve
Woo et al. 2009	Community health check	CS	1936 MF	NS	NS	Diseases of old age	Blood	PC	qPCR 6.1, 7.5	Only in men	Cat	Yes -ve

Clinical recruitment studies

	Study group	CS/Lo	# Sex	%Smoker	Age	Tissue	Extraction	Method COV %	Relation with age	Smoking measure	Effect
Aida et al. 2013	Upper GI endoscopy patients with alcohol dependency	CS	52 M	50+	60+	Oesophageal biopsies	n/a	qFISH NS	NS	Cat	No
Aida et al. 2015	Lung cancer cases and non-lung cancer autopsy cases	CS	89 MF	50+	60+	Lung	n/a	qFISH NS	No	Cat	Yes +ve
Atturu et	AAA cases and	CS	373	50+	60+	Blood	SO	TRF	No	Cat	No

al. 2010	controls		MF					NS			
Aulinas et al. 2015	Cushing's syndrome cases & controls	CS	154 MF	<25	45-59	Blood	PC	TRF NS	Yes	Cat	No
Balistreri et al. 2014	Sporadic TAA cases and controls	CS	160 MF	25-49	60+	Blood	Column	TRF NS	Yes	Cat	Yes -ve
Boyer et al. 2015	COPD cases plus controls	CS	273 MF	25-49	45-59	Blood	NS	qPCR NS	NS	Cat	No
Broberg et al. 2005	Bladder cancer cases plus controls	CS	156 MF	<25	60+	Buccal	Column	qPCR NS	Only in cases	Cat	No
Brouillette et al. 2003	Recruited from CCU registries plus controls	CS	383 MF	<25	45-59	Blood	SO	TRF 3.3	Yes	Both	No
Brouillette et al. 2008	Recruits with family history of coronary artery disease & controls	CS	104 MF	25-49	<45	Blood	NS	TRF NS	Only in cases	Both	No
Cronkhite et al. 2008	Idiopathic interstitial pneumonia cases & controls	CS	353 MF	50+	45-59	Blood	NS	qPCR & TRF <9, 13.6	Yes	Both	No
de Vusser	Donors of kidney	CS	217	25-49	45-59	Blood	SO	qPCR	Yes	Cat	No

et al. 2015	transplants & recipients		MF					NS			
Dlouha et al. 2016	Acute coronary syndrome cases plus controls	CS	1147 F	25-49	45-59	Blood	SO	qPCR 3.4-14.8	Yes	Cat	No
Du et al. 2015	Gastric cancer cases & controls	CS	2148 MF	50+	NS	Blood	NS	qPCR 10.16	Yes	Cat	Yes +ve in cases
Farhat et al. 2008	Patients undergoing CABG	CS	67 MF	25-49	60+	Endothelial cells	PC	TRF NS	NS	Cat	Yes +ve
Garland et al. 2014	Breast cancer cases	CS	392 F	<25	60+	Blood	NS	TRF NS	Yes	Cat	No
Getliffe et al. 2005	Ulcerative colitis & Crohn's disease cases	CS	34 MF	25-49	45-59	Blood	SO	qPCR NS	Yes	Cat	No
Haque et al. 2013	SLE cases & controls	CS	63 F	NS	45-59	Blood	Auto	qPCR NS	No	Cat	Yes +ve
Hartmann et al. 2010	Depression cases & controls	CS	74 MF	25-49	45-59	Blood	Column	TRF NS	Yes	Cat	No
Hou et al. 2009	Gastric adenocarcinoma	CS	716 MF	25-49	NS	Blood	NS	qPCR 8.1	Yes	Both	Yes -ve

	cases & controls										
Houben et al. 2009	COPD patients plus controls	CS	122 MF	NS	60+	Blood	SO	qPCR NS	Yes	Cont	No
Humphreys et al. 2012	Women reporting interpersonal violence & controls	CS	102 F	25-49	<45	Blood	NS	qPCR 3.5	Yes	Both	Yes +ve
Imam et al. 2012	HIV infected women and women enrolled in a mother/child HIV cohort study	CS	120 F	25-49	<45	Blood	Auto	qPCR NS	NS	Cat	No
Immonen et al. 2013	Age related macular degeneration cases & controls	CS	186 MF	25-49	60+	Blood	SO	TRF NS	No	Cat	No
Jang et al. 2008	Lung cancer cases & controls	CS	486 MF	25-49	45-59	Blood	Column	qPCR 7.5	Yes	Both	No
Jergović et al. 2014	Post-traumatic stress disorder cases & controls	CS	47 M	50+	45-59	Blood	PC	qPCR 11.21	No	Cat	Yes -ve in cases
Jiang et al. 2013	Stroke patients & siblings, & controls	CS	450 MF	25-49	45-59	Blood	Column	qPCR 6.7	Yes	Cat	No

Kato et al. 2016	Dialysis patients	Lo	59 MF	<25	60+	Blood	NS	qPCR NS	No	Cat	No
Leung et al. 2016	Mindfulness program for overweight pregnant women	Lo	65 F	25-49	<45	Blood	Column	qPCR 2.5	Yes	Cat	No
Lin et al. 2013	Part of a cancer screening study	CS	231 MF	25-49	45-59	Oesophageal cells	SO	qPCR 8.5	No	Cat	No
Liu et al. 2009	Gastric adenocarcinoma patients & controls	CS	774 MF	25-49	45-59	Blood	Column	qPCR NS	Yes	Cat	No
Liu et al. 2011	Hepatocellular carcinoma cases, hepatitis B virus related chronic liver disease cases & controls	CS	480 MF	25-49	45-59	Blood	Column	qPCR NS	Only in chronic liver disease cases	Cat	No
Liu et al. 2015	HIV positive cases & controls	CS	922 MF	50+	45-59	Blood	Column	qPCR NS	Yes	Both	Yes -ve
Magd et al. 2015	COPD cases & controls	CS	31 M	25-49	45-59	Blood	NS	qPCR NS	No	Both	Yes -ve

Matsumoto et al. 2015	Acromegaly cases	CS	46 MF	<25	45-59	Blood	SO	qPCR NS	No	Cat	No
Morlá et al. 2006	Smokers with & without COPD, plus controls	CS	76M	50+	45-59	Blood	n/a	FISH NS	Only in smokers	Cont	Yes -ve
Neuner et al. 2015	Blood donors	CS	343 MF	50+	<45	Blood	NS	qPCR 3.9	Yes	Both	No
Pathai et al. 2013	HIV positive cases & controls	CS	486 MF	NS	<45	Blood	Auto	qPCR NS	No	Cont	No
Pavanello et al. 2011	Alcohol dependents plus controls	CS	457 M	25-49	<45	Blood	IP	qPCR 3	Yes	Both	Yes +ve
Raymond et al. 2013	Idiopathic dilated cardiomyopathy & controls	CS	450 MF	<25	45-59	Blood	SO	qPCR NS	Yes	Cat	Yes -ve
Sadr et al. 2015	COPD cases & controls	CS	169 MF	50+	60+	Blood	Column	qPCR NS	NS	Cat	No
Sanchez-Espirdion et al. 2014	Lung cancer cases & controls	CS	2052 MF	50+	60+	Blood	Column	qPCR NS	Yes	Both	Yes -ve
Savale et	COPD cases &	CS	291	50+	60+	Blood	NS	qPCR	Yes	Both	No

al. 2009	controls		MF					NS			
Shen et al. 2012	Type 2 DM cases & controls	CS	4016 MF	<25	60+	Blood	NS	qPCR NS	Yes	Cat	No
Simon et al. 2006	MDD, BPD, & controls	CS	88 MF	25-49	45-59	Blood	NS	TRF NS	No	Cat	No
Srinivasa et al. 2014	HIV positive cases & controls	CS	142 M	NS	45-59	Blood	MB	qPCR 7	No	Cont	No
Steer et al. 2007	Rheumatoid arthritis cases & controls	CS	1327 MF	25-49	45-59	Blood	PC	TRF 0.92	NS	Cat	No
Suraweera et al. 2016	Colorectal cancer & colorectal adenoma cases	CS	324 MF	25-49	60+	Tumour, normal gut mucosa	Column	qPCR 7.5, 8.4	NS	Cat	No
Tentolouris et al. 2007	Type 2 DM cases	CS	84 M	<25	60+	Blood	NS	TRF NS	Yes	Cat	No
Tomita et al. 2010	COPD cases & controls	CS	44 MF	50+	45-59	Lung biopsies, alveolar macrophages	n/a	qFISH NS	NS	Both	Yes -ve
Unryn et al. 2006	Head & neck cancer cases & controls	CS	110 MF	NS	NS	Blood	PC	TRF NS	NS	Both	Yes -ve

Verde et al. 2016	Recruited from Occupational Medicine Committee	CS	145 MF	50+	<45	Blood	PC	qPCR NS	Yes	Cont	Yes -ve
Verde et al. 2015	Recruited from Occupational Medicine Committee	CS	147 MF	50+	<45	Blood	NS	qPCR NS	No	Cont	Yes -ve
Wang et al. 2014	Glioma cases & controls	CS	934 MF	<25	<45	Blood	IP	qPCR NS	Yes	Cat	No
Xiao et al. 2011	Type 2 DM cases & controls	CS	1797	<25	60+	Blood	NS	qPCR NS	Yes	Cat	Yes -ve
Zhou et al. 2015	Type 2 DM cases	CS	556	<25	45-59	Blood	Column	qPCR 7	No	Cat	No

As part of larger study

	Study	CS/ Lo	# Sex	% Smoker	Age	Healthy?	Tissue	Extra- ction	Method COV %	Relation with age	Smoking measure	Effect
Mui et al. 2009	Advair Biomarker in COPD study	CS	283 MF	NS	60+	All COPD cases	Blood	NS	qPCR NS	Yes	Both	No
Andreotti et al. 2015	Agricultural Health Study	CS	568 M	<25	45- 59	Yes	Blood	Unclear	qPCR 6.2	Yes	Cat	No

Hou et al. 2013		CS	1234 M	<25	NS	Yes	Buccal	IP	qPCR NS	Yes	Both	No
Shen et al. 2011	Alpha-Tocopherol, Beta-Carotene Cancer Prevention study	CS	458 M	50+	45-59	Lung cancer cases & controls	Blood	PC	qPCR 7	No	Cont	Yes -ve
Bekaert et al. 2007	Asklepios cohort study	CS	2509 MF	<25	45-59	Yes	Blood	SO	TRF 2.76	Yes	Both	No
Sen et al. 2014	Austrian Stroke Prevention Study	CS	786 MF	<25	60+	Yes	Blood	PC	qPCR NS	Yes	Cat	No
Saßenroth et al. 2015	Berlin Aging Study II	CS	809 MF	<25	60+	Yes	Blood	IP	qPCR NS	No	Cat	No
Aviv et al. 2009	Bogalusa Heart Study	Lo	635 MF	25-49	<45	Yes	Blood	SO	TRF 1.4	Yes	Cat	Yes -ve
Chen et al. 2011		Lo	271 MF	25-49	<45	Yes	Blood	NS	TRF NS	Yes	Cat	Yes -ve
Shen et al 2007	Breast Cancer Family Registry	CS	637 F	25-49	45-59	Breast cancer cases & controls	Blood	SO	qPCR 28	No	Both	No
Ehrlebenbach et	Bruneck Study	Lo	510 MF	<25	60+	Yes	Blood	IP	qPCR	At 1 TP	Cat	No

al. 2009									NS	out of 3		
Fitzpatrick et al. 2007	Cardiovascular Health Study	CS	419 MF	<25	60+	Yes	Blood	NS	TRF 1.5	Yes	Cat	No
Fitzpatrick et al. 2011		CS	1136 MF	<25	60+	Yes	Blood	NS	TRF 1.7	Yes	Cat	Yes (former shortest)
Sanders et al. 2012		CS	419 MF	<25	60+	Yes	Blood	NS	TRF 1.5	Yes	Cat	Yes -ve
Zanet et al. 2014	Children and women: AntiRetrovirals and the Mechanisms of Aging study	CS	395 MF	25-49	<45	HIV positive cases & controls	Blood	NS	qPCR NS	Yes	Both	Yes -ve
Chen et al. 2015	Chile Pediatric and Adult Sleep and Stress study	CS	89 MF	25-49	<45	Yes	Saliva	NS	qPCR <12	Yes	Cat	Yes -ve
Osler et al. 2016	Copenhagen Ageing Midlife Biobank study	CS	324 M	<25	NS	Yes	Blood	Auto	qPCR 5.8	NS	Cat	No

Weischer et al. 2014	Copenhagen City Heart study	Lo	4576 MF	25-49	45- 59	Yes	Blood	Column	qPCR 9.3	Yes	Both	Yes -ve
Rode et al. 2013	Copenhagen City Heart Study, Copenhagen General Population Study	CS	46396 MF	<25	45- 59	Yes	Blood	NS	qPCR NS	Yes	Both	Yes -ve
Rode et al. 2015		CS	64637 MF	50+	45- 59	Yes	Blood	Column	qPCR NS	Yes	Both	Yes -ve
Weischer et al. 2012		CS	9765 MF	25-49	45- 59	Yes	Blood	NS	qPCR 9	Yes	Cat	Yes -ve
Weischer et al. 2013		CS	47102 MF	25-49	60+	Yes	Blood	NS	qPCR 9.3	Yes	Both	Yes -ve
Rode et al. 2014	Copenhagen General Population Study	CS	55568 MF	50+	45- 59	Yes	Blood	Column	qPCR 9	Yes	Both	Yes -ve
Panayiotou et al. 2010	Cyprus Study	CS	762 MF	<25	60+	Yes	Blood	Column	qPCR NS	No	Cat	No
Kozlitina & Garcia 2012	Dallas Heart Study	CS	3157 MF	<25	45- 59	Yes	Blood	Auto	qPCR 4.09	Yes	Both	Yes -ve
Pellatt et al. 2012	Diet, Activity and Lifestyle Study	CS	1260 NS	NS	60+	Cancer cases & controls	Blood	NS	qPCR NS	Yes	Cont	Yes -ve
Müezzınler et	Epidemiological	Lo	961 MF	<25	NS	Yes	Blood	SO	qPCR	Yes	Both	Yes -ve

al. 2015	Study on the Chances of Prevention, Early Recognition, and Optimised Treatment of Chronic Diseases in the Older Population								NS			
Salpea et al. 2008	European Atherosclerosis Research Study II	CS	765 M	25-49	<45	Yes	Blood	SO	qPCR 5.6	No	Cat	No
Surtees et al. 2011	European Prospective	CS	4441 F	<25	60+	Yes	Blood	NS	qPCR 15	Yes	Cat	Yes -ve
Surtees et al. 2012	Investigation into Cancer Norfolk Population Study	CS	4441 F	<25	60+	Yes	Blood	NS	qPCR NS	Yes	Cat	Yes -ve
Marcon et al. 2012	European Prospective Project into	CS	56 MF	<25	NS	Yes	Blood	NS	TRF NS	Yes	Cat	No

	Cancer and Nutrition											
Nawrot et al. 2004	Flemish Study on Environment,	CS	271 MF	25-49	<45	Yes	Blood	NS	TRF <5	Yes	Cat	Yes -ve
Nawrot et al. 2010	Genes and Health Outcomes	CS	305 MF	25-49	<45	Yes	Blood	NS	TRF 1.7	Yes	Both	Yes -ve
Demissie et al. 2006	Framingham Heart Study	CS	327 M	<25	60+	Hypertensive cases & controls	Blood	NS	TRF NS	Yes	Cat	No
O'Donnell et al. 2008		CS	1062 MF	<25	45-59	Yes	Blood	NS	TRF 2.4	Yes	Cat	Yes -ve
Vasan et al. 2008		CS	1203 MF	<25	45-59	Yes	Blood	NS	TRF 2.4	Yes	Cat	Yes -ve
Verhulst et al. 2016	Genes, Familiar and Common Environment for the Development of Insulin Resistance,	Lo	676 MF	25-49	<45	Yes	Blood	NS	TRF 1.3	Yes	Cat	No

	Abdominal Adiposity, and Cardiovascular Risk Factors Study											
Wong et al. 2014	Harvard Boilermakers Longitudinal Study	CS	87 M	25-49	<45	Yes	Blood	NS	qPCR NS	NS	Cat	No
Ahola et al. 2012	Health 2000 Study	CS	2911 MF	25-49	<45	Yes	Blood	NS	qPCR 8.1	Yes	Cat	No
Kananen et al. 2010		CS	939 MF	NS	45-59	Anxiety disorder & controls	Blood	NS	qPCR NS	Yes	Cat	Yes -ve
Julin et al. 2015	Health Professionals Follow-up Study	CS	2483 M	50+	60+	Yes	Blood	Column	qPCR 11.8-16	Yes	Both	Yes -ve
Adler et al. 2013	Health, Aging and Body Composition Study	CS	2599 MF	50+	60+	Yes	Blood	NS	qPCR NS	Yes	Cat	Yes -ve
O'Donovan et al. 2011		CS	1962 MF	<25	60+	Yes	Blood	NS	qPCR 4	Yes	Cat	Yes -ve
Sanders et al.		CS	2750	NS	NS	Yes	Blood	NS	qPCR	Yes	Cat	Yes -ve

2009			MF						5.8			
Sanders et al. 2011	Health, Aging and Body Composition study; ARMA study	CS	4208 MF	<25	60+	Cataract	Blood	NS	qPCR 5.8	Yes	Cat	Yes -ve
Strandberg et al. 2011	Helsinki Businessmen Study	CS	622 M	<25	60+	Yes	Blood	NS	TRF 3.7	No	Cat	Yes -ve
Kark et al. 2012	Jerusalem Lipid Research Clinic Prevalence Study	Lo	609 MF	25-49	<45	Yes	Blood	SO	TRF 2.1-2.2	NS	Both	Yes -ve
Benetos et al. 2013	Jerusalem Lipid Research Clinic, Bogalusa Heart Study, Evolution de la Rigidite Arterielle, Longitudinal Study of Aging Danish Twins	Lo	1156 MF	25-49	<45	Yes	Blood	NS	TRF 2.3	NS	Cat	Yes -ve

Shin et al. 2015	Korean Genome Epidemiology Study	CS	2128 MF	<25	45- 59	Yes	Blood	Column	qPCR NS	Yes	Cat	No
Fyhrquist et al. 2011	Lifestyle Interventions and Independence for Elders study	CS	1271 MF	NS	60+	Hypertension & left ventricular hypertrophy	Blood	SO	TRF NS	In males	NS	No
Fyhrquist et al. 2013		CS	1249 MF	25-49	60+	Hypertension & left ventricular hypertrophy cases	Blood	SO	TRF NS	Yes	Cat	Yes -ve
Shen et al. 2009	Long Island Breast Cancer Study Project	CS	2169 F	NS	NS	Breast ca patients	Blood	PC	qPCR NS	Yes	Both	No
Bischoff et al. 2006	Longitudinal Danish Centenarian study	CS	812 MF	NS	60+	Yes	Blood	NS	TRF 12	NS	Cat	No
Bendix et al. 2014	Longitudinal Study of Aging Danish	CS	439 MF	25-49	60+	Yes	Blood	NS	TRF 3.4	Yes	Cat	Yes -ve

	Twins											
Lee et al. 2012	Lung Health study	CS	4271 MF	50+	45- 59	No- airflow limitation smokers	Blood	NS	qPCR NS	No	Both	No
Epel et al. 2009	MacArthur Health Aging Study	Lo	236 MF	<25	60+	Yes	Blood	SO	qPCR NS	No	Cont	No
Nordfjäll et al. 2008	Malmo Diet and Cancer Cohort, MONICA project	CS	1502 MF	<25	45- 59	Yes	Blood	NS	qPCR NS	Yes	Cont	No
van der Harst et al. 2007	Metoprolol CR/XL Randomized Intervention Trial in Congestive Heart Failure plus additional CHF patients & controls	CS	620 MF	<25	60+	No- CHF	Blood	NS	qPCR NS	Yes	Cat	No
Raschenberger et al. 2015	Mild to Moderate Kidney Disease Study & Chronic	CS	1055 MF	<25	60+	No- CKD	Blood	Auto	qPCR 7.6-7.9	Yes	Cat	Yes -ve

	Renal Insufficiency Standards Implementation Study											
Bendix et al. 2013	MONICA 1	Lo	1765 MF	50+	<45	Yes	Blood	Column	qPCR 7.8	Yes	Cat	Yes -ve
Ellehoj et al. 2016	MONICA project	CS	1397 MF	50+	<45	CVD, IHD, stroke patients	Blood	NS	qPCR 5.8	NS	Cat	No
Carroll et al. 2013	Multi-ethnic study of atherosclerosis	CS	963 MF	NS	60+	Yes	Blood	NS	qPCR 7	Yes	Cont	Yes -ve
Carroll et al. 2013		CS	948 MF	NS	60+	Yes	Blood	NS	qPCR 7	Yes	Cont	Yes -ve
Needham et al. 2014		CS	851 MF	NS	60+	CVD	Blood	NS	qPCR 7	NS	Cont	Yes -ve
Roux et al. 2009		CS	981 MF	NS	60+	Yes	Blood	NS	qPCR 7	Yes	Cont	Yes -ve
Ahrens et al. 2016	National Health and Nutrition Examination study	CS	1503 F	<25	NS	Yes	Blood	NS	qPCR NS	NS	Cat	No
Loprinzi 2015	Examination study	Lo	1764	NS	<45	Yes	Blood	NS	qPCR	Yes	Cat	No

			MF						<7			
Needham et al. 2013		CS	5360 MF	25-49	45-59	Yes	Blood	NS	qPCR 4.4	Yes	Cont	Yes -ve
Needham et al. 2015		CS	3091 MF	<25	NS	Yes	Blood	NS	qPCR NS	Yes	Cat	No
Patel et al. 2016		CS	3567 MF	25-49	45-59	Yes	Blood	NS	qPCR NS	Yes	Both	Yes -ve
Wulaningsih et al. 2016		CS	6456 MF	<25	45-59	Yes	Blood	NS	qPCR 6.5	Yes	Both	Yes -ve
Zota et al. 2014		CS	6796 MF	25-49	NS	Yes	Blood	SO	qPCR NS	Yes	Cont	Yes -ve
Risques et al. 2010	National Long Term Care Survey	CS	624 MF	<25	60+	Some disabled	Blood	Auto	qPCR 7	No	Cat	No
Révész et al. 2016		CS	2750 MF	NS	<45	Depression, anxiety	Blood	NS	qPCR NS	NS	Cont	Yes -ve
Park et al. 2015	Netherlands Study of Depression and Anxiety	CS	2901 MF	25-49	<45	Yes	Blood	NS	qPCR NS	NS	Cat	Yes -ve
Dóra Révész et al. 2016		Lo	2921 MF	NS	<45	No- mental healthcare recipients	Blood	NS	qPCR NS	Yes	Cont	Yes -ve

Verhoeven et al. 2015		CS	2936 MF	25-49	<45	Depression, anxiety	Blood	NS	qPCR 4.6	Yes	Cat	Yes -ve
Verhoeven et al. 2016		Lo	2936 MF	25-49	<45	Depression, anxiety	Blood	NS	qPCR 4.6	Yes	Cat	Yes -ve
Terry et al. 2012	New England case-control study; Nurses' Health study	CS	947 F	<25	NS	Yes (controls)	Blood	NS	qPCR NS	Yes	Cat	Yes, former smokers shorter
Collerton et al. 2007	Newcastle 85+ Study	CS	84 MF	<25	60+	Yes	Blood	NS	qPCR NS	No	Cat	No
Adams et al. 2007	Newcastle Thousand Families Study	CS	318 MF	NS	45-59	Yes	Blood	NS	qPCR NS	NS	Cont	No
Pearce et al. 2012		CS	318 MF	25-49	NS	Yes	Blood	NS	qPCR 6.2	NS	Cat	Yes -ve
Hou et al. 2015	Normative Aging Study	Lo	579 M	<25	60+	Yes	Buccal	NS	qPCR NS	Yes	Both	No
Cassidy et al. 2010	Nurses' Health Study	CS	2284 F	<25	45-59	Yes	Blood	NS	qPCR 14-16	Yes	Both	No
Crous-Bou et al. 2014		CS	4676 F	NS	45-59	Yes	Blood	Column	qPCR <18	Yes	Cont	Yes -ve

De Vivo et al. 2009		CS	2269 F	NS	45- 59	Breast ca cases, controls	Blood	Column	qPCR 16.3	Yes	Cont	No
Du et al. 2012		CS	7684 F	<25	45- 59	Yes	Blood	Column	qPCR <17	Yes	Cat	Yes -ve
Liang et al. 2011		CS	4117 F	<25	45- 59	Yes	Blood	Column	qPCR NS	Yes	Both	No
Liu et al. 2013		CS	4604 F	NS	45- 59	Yes	Blood	NS	qPCR 14-16.3	Yes	Cont	No
Schürks et al. 2013		CS	1008 F	<25	60+	Ischaemic stroke	Blood	Column	qPCR 22	No	Cat	No
Sun et al. 2012		CS	5862 F	<25	45- 59	Yes	Blood	Column	qPCR 10.8-16	Yes	Cat	Yes -ve
Liu et al. 2016		CS	4780 F		45- 59	Yes	Blood	NS	qPCR <18	Yes	Cont	Yes -ve
McGrath et al. 2007	Nurses' Health Study and Health Professionals Follow-up Study	CS	376 MF	<25	60+	Bladder cancer in 184 subjects	Blood	Column	qPCR NS	No	Both	Yes -ve (HPFS only)
Prescott et al. 2010	Nurses' Health Study and	CS	1181 F	NS	45- 59	Yes	Blood	NS	qPCR 14	Yes	Both	No

	Women's Health Study											
Mason et al. 2015	Nurses' Health Study II	CS	1135 F	NS	45-59	Yes	Blood	Column	qPCR 11.5	NS	Cat	Yes -ve
Svensson et al. 2014	Osteoporotic Fractures in Men Study	CS	2744 M	<25	60+	Yes	Blood	NS	qPCR 10.7	No	Cat	No
Yu et al. 2015		CS	2006 MF	<25	60+	Yes	Blood	PC	qPCR 11.1	Yes, in men	Cat	No
Mather et al. 2010	PATH Through Life Project	CS	646 MF	<25	45-59	Yes	Blood	Column	qPCR 3.44, 8.82	No, but diff between 2 age cohorts	Cat	No
Zee et al. 2009	Physicians Health Study	CS	497 M	<25	45-59	Colorectal ca	Blood	Column	qPCR <2	Yes	Cat	No
Zee et al. 2010		CS	518 M	<25	60+	Stroke patients	Blood	Column	qPCR <2	Yes	Cat	No
Huzen et al. 2014	Prevention of Renal and	Lo	8074 MF	25-49	45-59	Yes	Blood	Column	qPCR 4.5	Yes	Both	Yes -ve
Kingma et al.	Vascular End-	Lo	895 MF	<25	45-	Yes	Blood	Column	qPCR	Yes	Cont	No

2012	Stage Disease study				59				3.9			
van Ockenburg et al. 2014		Lo	2156 MF	25-49	45-59	Yes	Blood	NS	qPCR 4.5	Yes	Both	No
van Ockenburg et al. 2015		Lo	1094 MF	<25	45-59	Yes	Blood	Column	qPCR 4.5	Yes	Both	Yes -ve (16-20 CPD)
Fernández-Alvira et al. 2016	Progression of Early Subclinical Atherosclerosis study	CS	1459 MF	NS	45-59	Subclinical atherosclerosis	Blood	n/a	HT-QFISH 5.6	Yes	Both	No
Baragetti et al. 2015	Progressione della Lesione Intimale Corotidea Study	Lo	768 MF	25-49	45-59	Yes	Blood	SO	qPCR 7.3	Yes	Cat	Yes -ve
Mirabello et al. 2009	Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial	CS	1661 M	50+	NS	Prostate cancer pts and controls	Blood	Column	qPCR NS	Yes	Cont	Yes -ve
Shiels et al. 2011	Psychological, social and	CS	382 NS	NS	NS	Yes	Blood	NS	qPCR NS	Yes	Cat	Yes -ve

	biological determinants of ill health study											
Hardikar et al. 2015	Seattle Barrett's esophagus study	CS	295 MF	50+	60+	Barrett's oesophagus patients	Blood	NS	qPCR 7	Yes	Both	Yes -ve
Risques et al. 2007		CS	287 MF	50+	NS	Metaplasia in oesophageal biopsies	Blood	SO	qPCR 7	Yes	Cat	No
Rane et al. 2015	Singapore Chinese Health Study	CS	90 MF	25-49	45-59	Yes	Blood	Column	TRF NS	No	Cat	No
Aviv et al. 2006	St. Thomas' (Twins UK) Adult Twin Registry	CS	1517 F	<25	45-59	Yes	Blood	NS	TRF NS	Yes	Cat	Yes -ve
Barwell et al. 2007		CS	1768 F	NS	45-59	Breast cancer cases & controls	Blood	NS	TRF 1.4	NS	Cat	Yes -ve
Cherkas et al. 2006		CS	1552 F	<25	45-59	Yes	Blood	NS	TRF NS	Yes	Cat	Yes -ve
Cherkas et al. 2008		CS	2401 MF	<25	45-59	Yes	Blood	NS	TRF NS	Yes	Cat	Yes -ve

Valdes et al. 2005		CS	1103 F	<25	45-59	Yes	Blood	NS	TRF NS	Yes	Both	Yes -ve
Chen et al. 2014	Strong Heart Family study	CS	3256 MF	25-49	<45	Yes	Blood	NS	qPCR 6.9	Yes	Cat	No
Zhao et al. 2014		CS	2328 MF	25-49	<45	Yes	Blood	NS	qPCR 6.9	Yes	Cat	No
Borghini et al. 2016	Studies on exposures to inorganic arsenic	CS	241 MF	25-49	<45	Yes	Blood	Column	qPCR NS	Yes	Cat	No
Bakaysa et al. 2007	Swedish Twin Registry	CS	350 MF	<25	60+	Yes	Blood	NS	TRF NS	Yes	Cat	No
von Känel et al. 2015	Sympathetic Activity and Ambulatory Blood Pressure in Africans study	CS	341 MF	<25	45-59	Yes	Blood	Column	qPCR 4.8	Yes	Cat	No
Masi et al. 2012	Ten Towns Heart Health study	CS	1080 MF	<25	<45	Yes	Blood	SO	qPCR 3.27	Yes	Cat	No
Farzaneh-Far et al. 2010	The Heart and Soul study	Lo	608 MF	<25	60+	CVD patients	Blood	IP	qPCR 3.7	Yes	Cat	No

Harris et al. 2006	The Lothian Birth Cohort 1921	CS	190 MF	<25	60+	Yes	Blood	NS	qPCR NS	NS	Both	No
Harris et al. 2012	The Lothian Birth Cohort 1936	CS	1048 MF	<25	60+	Yes	Blood	NS	qPCR 5.12	No	Both	No
Kim et al. 2011	The Sister Study	CS	1077 F	<25	45- 59	Breast cancer cases & controls	Blood	Auto	qPCR 11	Yes	Cat	No
Parks et al. 2009		CS	740 F	<25	NS	Yes	Blood	NS	qPCR 8.5	Yes	Cat	Yes -ve
Parks et al. 2011		CS	647 F	<25	45- 59	Yes	Blood	NS	qPCR 8.5	NS	Cat	Yes -ve
Masi et al. 2016	University College Diabetes and Cardiovascular Disease Study	CS	489 MF	<25	60+	Diabetic clinic attendees	Blood	SO	qPCR 5.6	Yes	Cat	No
Salpea et al. 2010	University College Diabetes and Cardiovascular Disease Study, European	CS	1017 MF cases M controls	50+	45- 59	Cases type 2 DM	Blood	SO	qPCR 5.6	Yes	Cat	No

	Atherosclerosis Research Study II, Hypercoagulability and Impaired Fibrinolytic Function Mechanisms predisposing to myocardial infarction study											
Gu et al. 2015	Washington Heights-Inwood Community Aging Project	CS	1743 MF	25-49	60+	Yes	Blood	NS	qPCR 5-8	Yes	Cat	No
Batty et al. 2009	West of Scotland Coronary Prevention Study	CS	1542 M	NS	NS	High risk for coronary heart disease	Blood	NS	qPCR NS	Yes	Cat	No
Brouillette et al. 2007	West of Scotland Primary Prevention Study	CS	1542 M	50+	45-59	Coronary heart disease cases &	Blood	NS	qPCR NS	Only in controls	Cat	No

						controls						
Phillips et al. 2013	West of Scotland Twenty-07 Study	Lo	1063 MF	<25	45- 59	Yes	Blood	NS	qPCR NS	Yes	Cat	No
Jackowska et al. 2012	Whitehall II epidemiological study	CS	434 MF	<25	60+	Yes	Blood	NS	qPCR NS	No	Cat	Yes -ve in men
Steptoe et al. 2011		CS	448 MF	<25	60+	Yes	Blood	Auto	qPCR 3.3	NS	Cat	No
Song et al. 2013	Women's' Health Initiative Observational Study	CS	4029 F	<25	60+	Yes	Blood	NS	qPCR 5.7	Yes	Cat	No
Houben et al. 2011	Zutphen Elderly Study	Lo	203 M	25-49	60+	Yes	Blood	Column	qPCR NS	No	Cat	No

Appendix 10: Chapter 5 additional analyses

Weight was significantly positively associated with LRTL1 ($F=10.1_{(1,39)}$, $R^2=0.21$, $p=0.003$) and BRTL2 ($F=19.37_{(1,18)}$, $R^2=0.52$, $p<0.001$), less strongly with BRTL1 ($F=6.75_{(1,38)}$, $R^2=0.15$, $p=0.01$) and not significantly related to LRTL2 ($F=1.71_{(1,22)}$, $R^2=0.07$, $p=0.21$) (Figure A10.1). Interestingly, there was a strong positive relationship between CMRTL and dog weight ($F=159.6_{(1,34)}$, $R^2=0.84$, $p<0.001$, Figure A10.2A). Linear models of weight to ERTL and VDRTL were also significant with positive beta coefficients (ERTL $F=33.19_{(1,32)}$, $R^2=0.51$, $p<0.001$; VDRTL $F=9.01_{(1,33)}$, $R^2=0.21$, $p=0.005$; Figure A10.2B/C). There were also negative relationships between the difference in LRTL1 and CMRTL, and dog weight ($AIC=112.68$, $p<0.001$, Figure A10.3A) and the difference between LRTL2 and CMRTL and dog weight ($F=20.9_{(1,21)}$, $R^2=0.50$, $p<0.001$, Figure A10.3B), indicating larger dogs had an increased difference between their CMRTL and LRTL measures. In addition, there was a positive relationship between the rate of change between CMRTL and LRTL1 and weight ($AIC=-32.56$, $p=0.002$, Figure A10.3C), as well as the rate of change between CMRTL and LRTL2 and dog weight ($F=13.06_{(1,21)}$, $R^2=0.38$, $p=0.002$, Figure A10.3D), indicating a faster rate of change in larger dogs. In terms of excess body weight, there was a significant negative relationship between the difference in LRTL2 and CMRTL and kilogram weight gain (adjusted for weight gain due to growth) ($F=11.04_{(1,21)}$, $R^2=0.34$, $p=0.003$, Figure A10.4B). In addition, BRTL2 increased with additional weight gain above weight gain due to growth ($F=10.25_{(1,18)}$, $R^2=0.36$, $p=0.005$, Figure A10.4A).

Like weight, height was positively associated with both LRTL1 ($F=11.39_{(1,39)}$, $R^2=0.23$, $p=0.002$), BRTL1 ($F=7.73_{(1,38)}$, $R^2=0.17$, $p=0.008$) and BRTL2 ($F=20.2_{(1,18)}$, $R^2=0.53$, $p<0.001$), but not LRTL2 ($F=1.85_{(1,22)}$, $R^2=0.08$, $p=0.19$). Height was also positively associated with CMRTL ($F=169.8_{(1,34)}$, $R^2=0.83$, $p<0.001$), ERTL ($F=35.47_{(1,32)}$, $R^2=0.53$, $p<0.001$) and VDRTL ($R^2=0.20$, $p=0.006$). Likewise, with weight, taller dogs had a greater difference between LRTL1 and CMRTL ($AIC=111.51$, $p<0.001$) and LRTL2 and CMRTL ($R^2=0.53$, $p<0.001$), as well as a faster rate of change between CMRTL and LRTL1 ($AIC=-35.33$, $p<0.001$) and CMRTL and LRTL2 ($R^2=0.41$, $p<0.001$) in taller dogs.

The associations between sex and LRTL1 and sex and BRTL1 were only briefly examined, with there being just 5 females at time point 1 and 1 female returning for follow up. Paired t-tests showed there was no difference in LRTL1 or BRTL1 between the sexes ($p=0.67$ and $p=0.52$, respectively) (Figure A10.5).

Breed was a difficult variable to examine given the small numbers of each breed. Initially, the dogs were divided into 4 groups: spaniel/spaniel cross, terrier/terrier cross, collie/collie cross and other. The “other” group was excluded and one-way ANOVAs performed which showed no significant difference in LRTL1 ($p=0.07$), BRTL1 ($p=0.13$), CMRTL ($p=0.47$), and LRTL2 ($p=0.08$), but there was a significant difference in BRTL2 between the groups ($p=0.03$) (Figures A10.6 & A10.7). It was noted during the analyses that the collie group seemed to have longer LRTL and BRTL measures, and thus all the dogs were divided into 2 groups: collie/collie cross and other. Paired t-tests indicated a statistically significant difference in LRTL1 ($p=0.03$) and BRTL1 ($p=0.04$) between the 2 groups, but while mean LRTL2 and BRTL2 were longer in the collie group, the differences were not statistically significant ($p=0.23$ and $p=0.14$, respectively). There were also no statistically significant differences in CMRTL ($p=0.27$), ERTL ($p=0.18$) or VDRTL ($p=0.17$), however, in the collie group the mean value was higher for all these tissues as well.

CMRTL was positively associated with breed life expectancy ($F=100.5_{(1,24)}$, $R^2=0.81$, $p<0.001$, Figure A10.8C), despite there being a strong positive relationship between weight and CMRTL, and there being a negative trend between lifespan and weight (Spearman’s $\rho=-0.21$, $p=0.25$). As with increasing weight, there was a larger difference between LRTL1 and CMRTL with increasing life expectancy (AIC=78.0, $p<0.001$, Figure A10.8D). Breed life expectancy was also significantly related to both LRTL1 ($F=6.26_{(1,29)}$, $R^2=0.18$, $p=0.02$) and LRTL2 ($F=6.97_{(1,13)}$, $R^2=0.35$, $p=0.02$) (Figure A10.8A&B).

Dogs were divided into two groups based upon whether their LRTL measurements lengthened (L) or shortened (S) between time points 1 and 2. The dog which was classed as a maintainer was not included in this analysis. Table A10-1 shows the results of a series of statistical tests examining whether biochemical and haematological measures were different in groups L or S, and also if the changes in the biochemical and haematological measures between the two time points were different between the two groups. Mean cell haemoglobin concentration was significantly higher in dogs in the S group than the L group at time point 1 ($p < 0.001$). In addition, mean cell volume was significantly different between the groups at both time points, with the L group having higher average values at both points. The change in monocyte count between the two time points was significantly different between the two groups, with the L group having little change and the S group having higher monocyte counts at time point 1 than 2.

Table A10-1 P values of pairwise testing between LRTL shorteners and elongators of measures at time point 1 (TP1), time point 2 (TP2) and the change in measures between the two time points.

Significance level denoted by * <0.05, **<0.01, *<0.001**

	TP 1	TP2	TP1 – TP2
Sodium concentration	0.37	0.40	0.79
Potassium concentration	0.22	0.92	1
Sodium:potassium ratio	0.15	0.67	0.27
Chloride concentration	0.89	0.11	0.33
Calcium concentration	0.21	0.71	0.83
Phosphate concentration	0.73	0.08	0.17
Urea concentration	0.89	0.54	0.49
Creatinine concentration	0.78	0.77	0.53
Cholesterol concentration	0.29	0.54	0.86
Triglyceride concentration	0.08	0.22	0.74
Total bilirubin concentration	0.26	0.17	0.53
ALK Phosphatase concentration	0.76	0.86	0.90
AST concentration	0.37	0.54	0.85
ALT concentration	0.91	0.06	0.29
GGT concentration	0.19	0.09	0.36
Total protein concentration	0.47	0.37	0.82
Albumin concentration	0.40	0.06	0.90
Globulin concentration	0.79	0.75	0.57
Albumin:globulin ratio	0.57	0.23	0.48
Red cell count	0.85	0.27	0.28
Haemoglobin concentration	0.63	0.58	0.43
Haematocrit	0.35	0.69	0.32
Mean cell volume	0.03*	0.02*	0.76
Mean cell haemoglobin	0.54	0.05*	0.19
Mean cell haemoglobin concentration	<0.001***	0.49	0.06
Red cell distribution width	0.08	0.56	0.08

White cell count	0.73	0.53	0.66
Neutrophil count	0.37	0.79	0.32
Lymphocyte count	0.40	0.21	0.60
Neutrophil:lymphocyte ratio	0.30	0.60	0.40
Monocyte count	0.22	0.21	0.02*
Lymphocyte:monocyte ratio	0.83	0.19	0.10
Eosinophil count	0.57	0.24	0.19
Platelet count	0.59	0.24	0.28
Mean platelet volume	0.91	0.92	0.97
Plateletcrit	0.35	0.06	0.49
Platelet distribution width	0.81	0.87	0.61

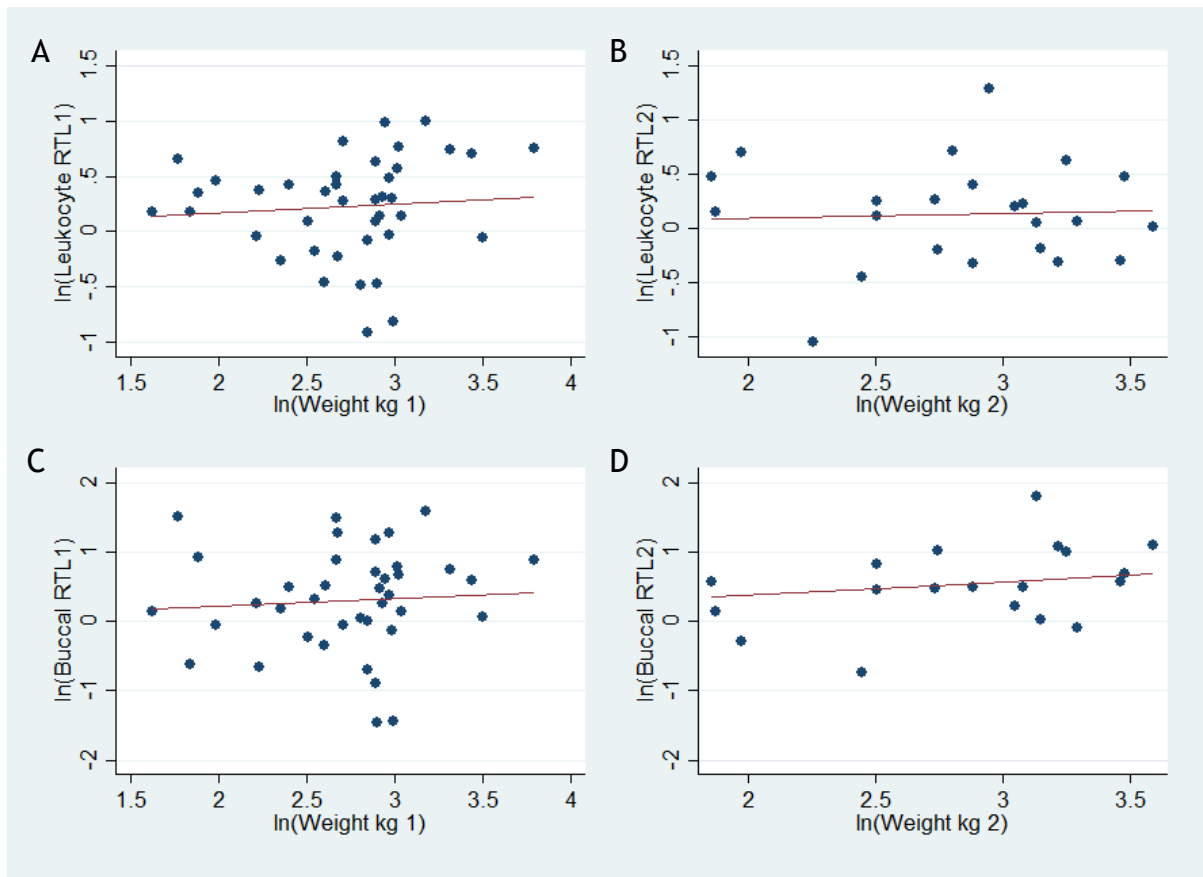


Figure A10.1 Scatterplots with regression line of leukocyte and buccal RTL by dog weight
 Natural log of leukocyte RTL and natural log of dog weight at time point 1 (A) ($R^2=0.21$, $p=0.003$); natural log of leukocyte RTL and natural log of dog weight at time point 2 (B) ($R^2=0.07$, $p=0.21$); natural log of buccal RTL and dog weight at time point 1 (C) ($R^2=0.15$, $p=0.01$); natural log of buccal RTL and dog weight at time point 2 (D) ($R^2=0.52$, $p<0.001$)

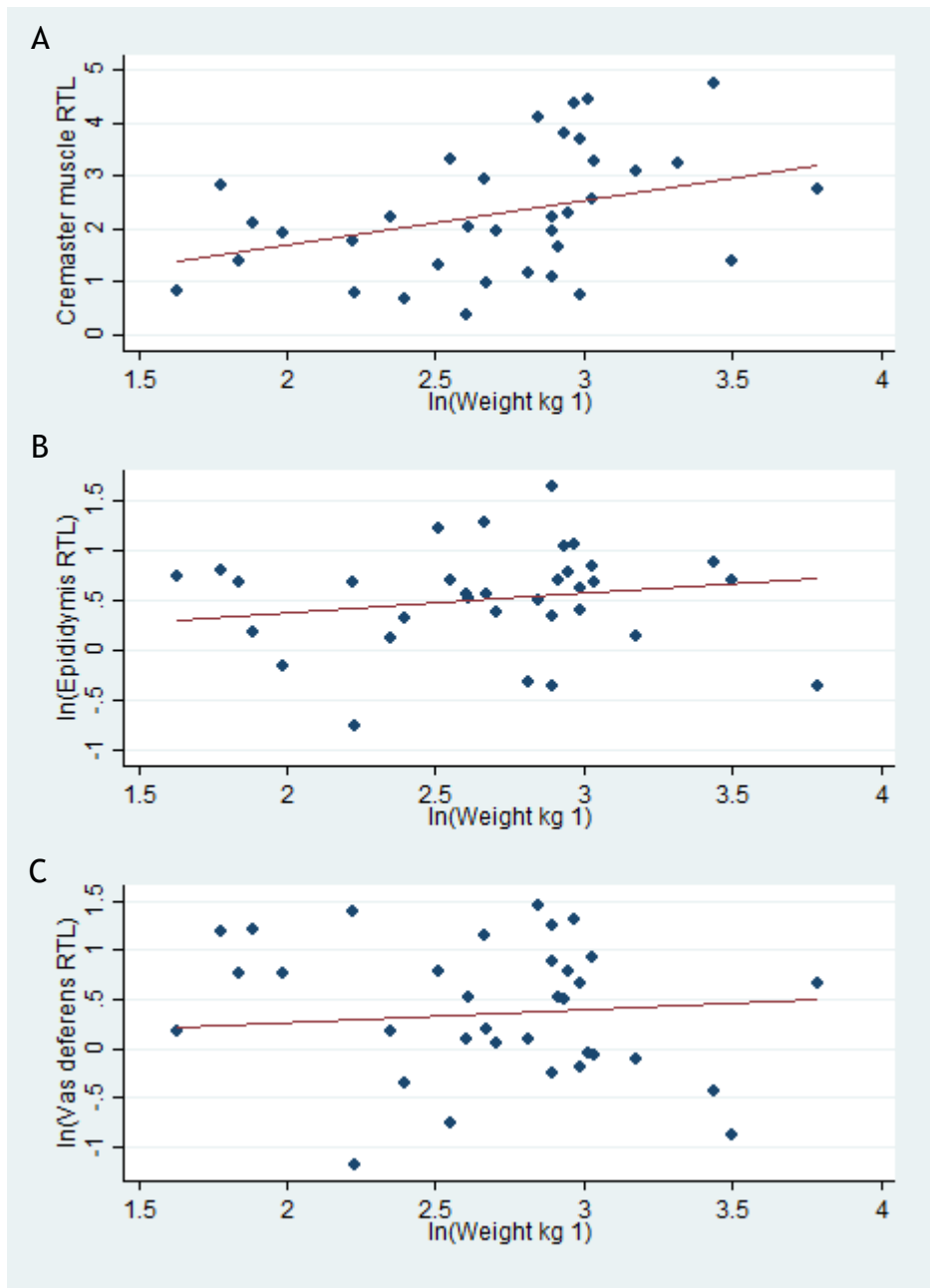


Figure A10.2 Scatterplots with regression line of cremaster muscle, epididymis and vas deferens RTL by dog weight
 Cremaster muscle RTL and natural log of dog weight (A) ($R^2=0.82$, $p<0.001$); natural log of epididymis RTL and natural log of dog weight (B) ($R^2=0.51$, $p<0.001$); and natural log of vas deferens RTL and natural log of dog weight (C) ($R^2=0.13$, $p=0.005$)

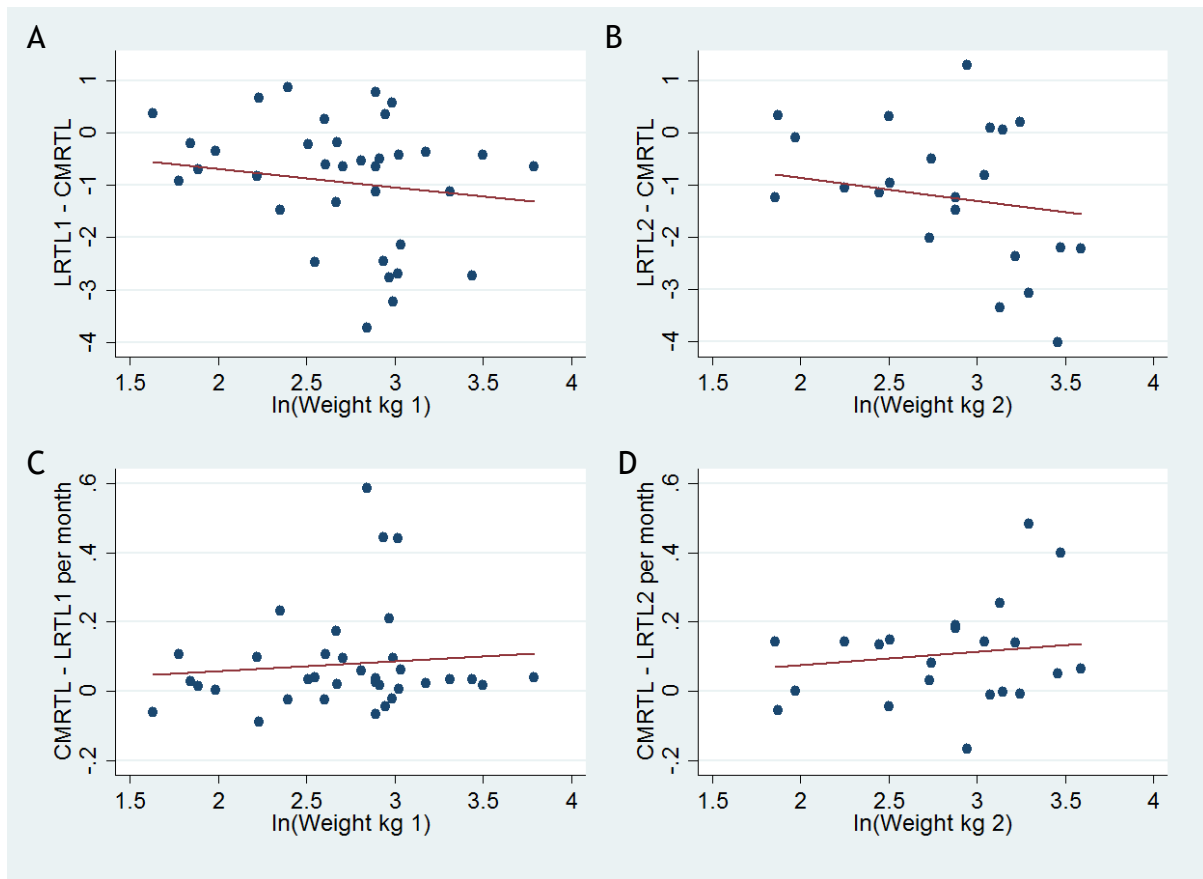


Figure A10.3 Scatterplots with regression line of differences in tissue RTL by dog weight
 The difference in leukocyte RTL and cremaster muscle and natural log of dog weight at time point 1 (A) (AIC=112.68, $p<0.001$); difference in leukocyte RTL and cremaster muscle and dog weight at time point 2 (B) ($R^2=0.50$, $p<0.001$); change in difference between cremaster muscle RTL and leukocyte RTL from time point 1 per month and dog weight at time point 1 (C) (AIC=-32.56, $p=0.002$); change in difference between cremaster muscle RTL and leukocyte RTL from time point 2 per month and dog weight at time point 2 (D) ($R^2=0.38$, $p=0.002$)

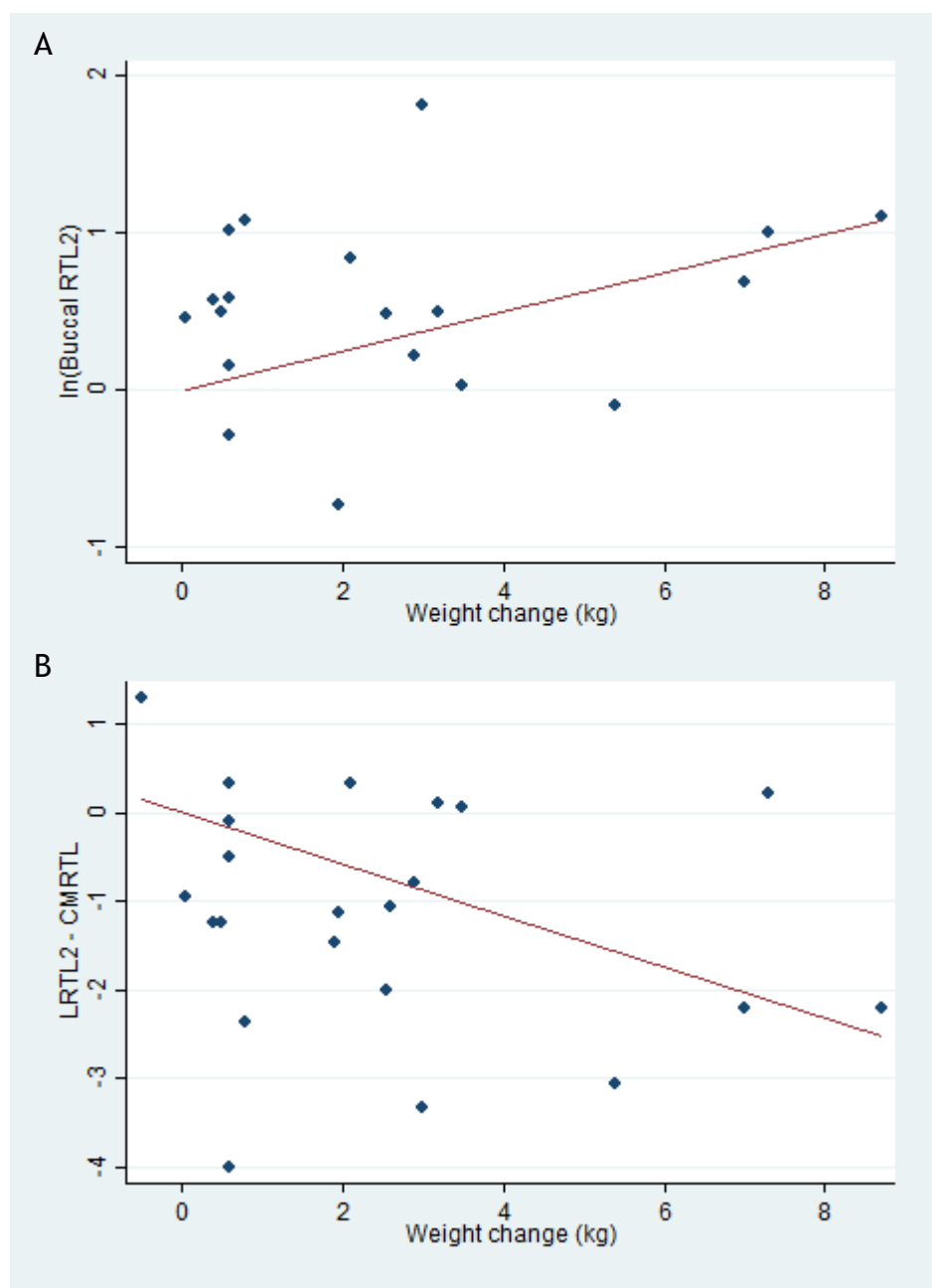


Figure A10.4 Scatterplots with regression line of buccal RTL and the difference between leukocyte and cremaster RTL at time point 2 by change in dog weight
 Natural log of buccal RTL at time point 2 and weight change between the two time points, adjusted for expected weight gain due to growth (A) ($R^2=0.36$, $p=0.005$); leukocyte RTL 2 minus cremaster muscle RTL and adjusted weight change (B) ($R^2=0.34$, $p=0.003$)

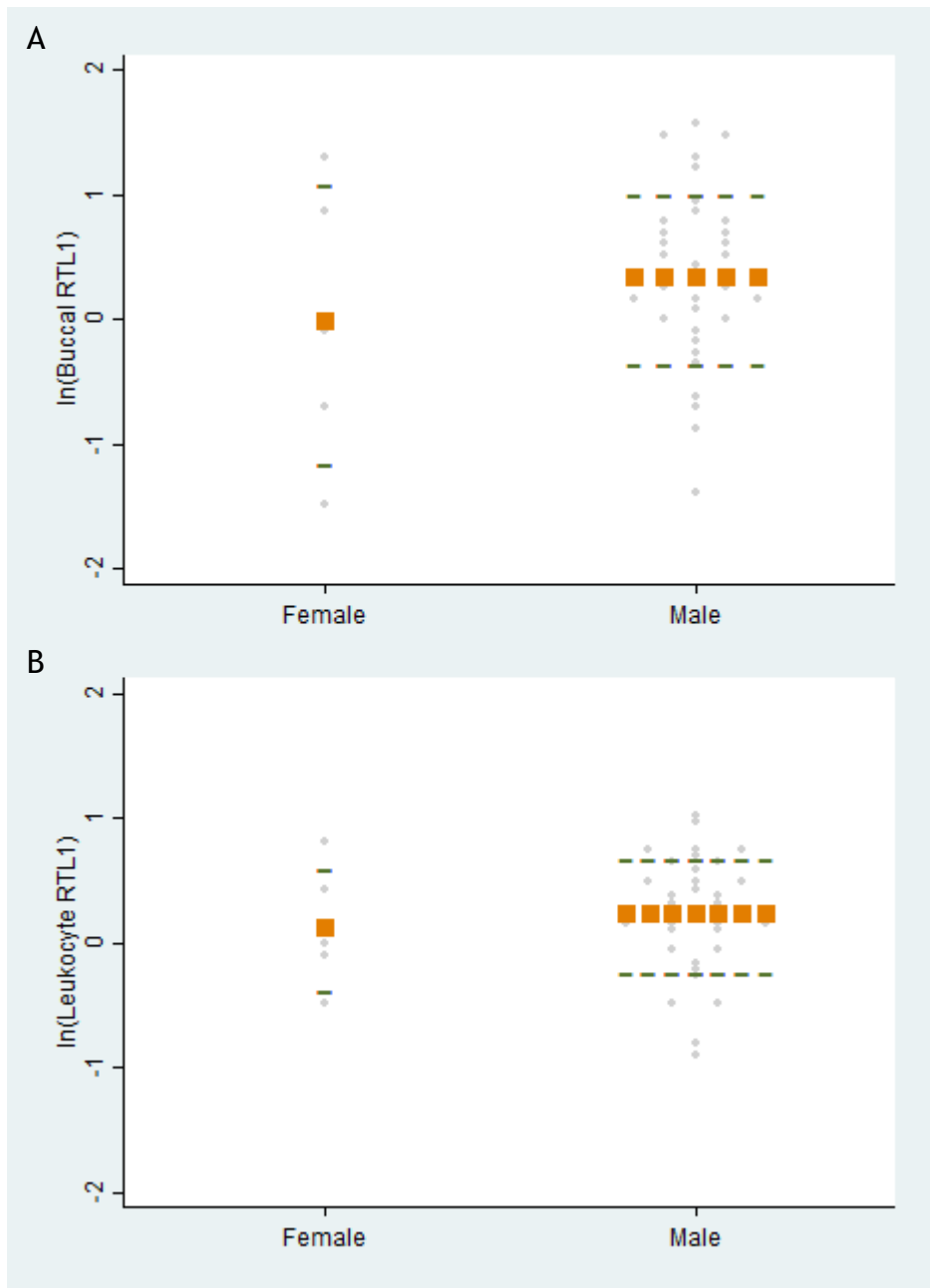


Figure A10.5 Dot plots of natural log of buccal RTL1 (A) and leukocyte RTL1 (B) by sex. The orange squares represent the mean value and green dashes the 95% confidence intervals. No significant difference was found in buccal RTL1 ($p=0.52$) or leukocyte RTL1 ($p=0.67$) between the sexes.

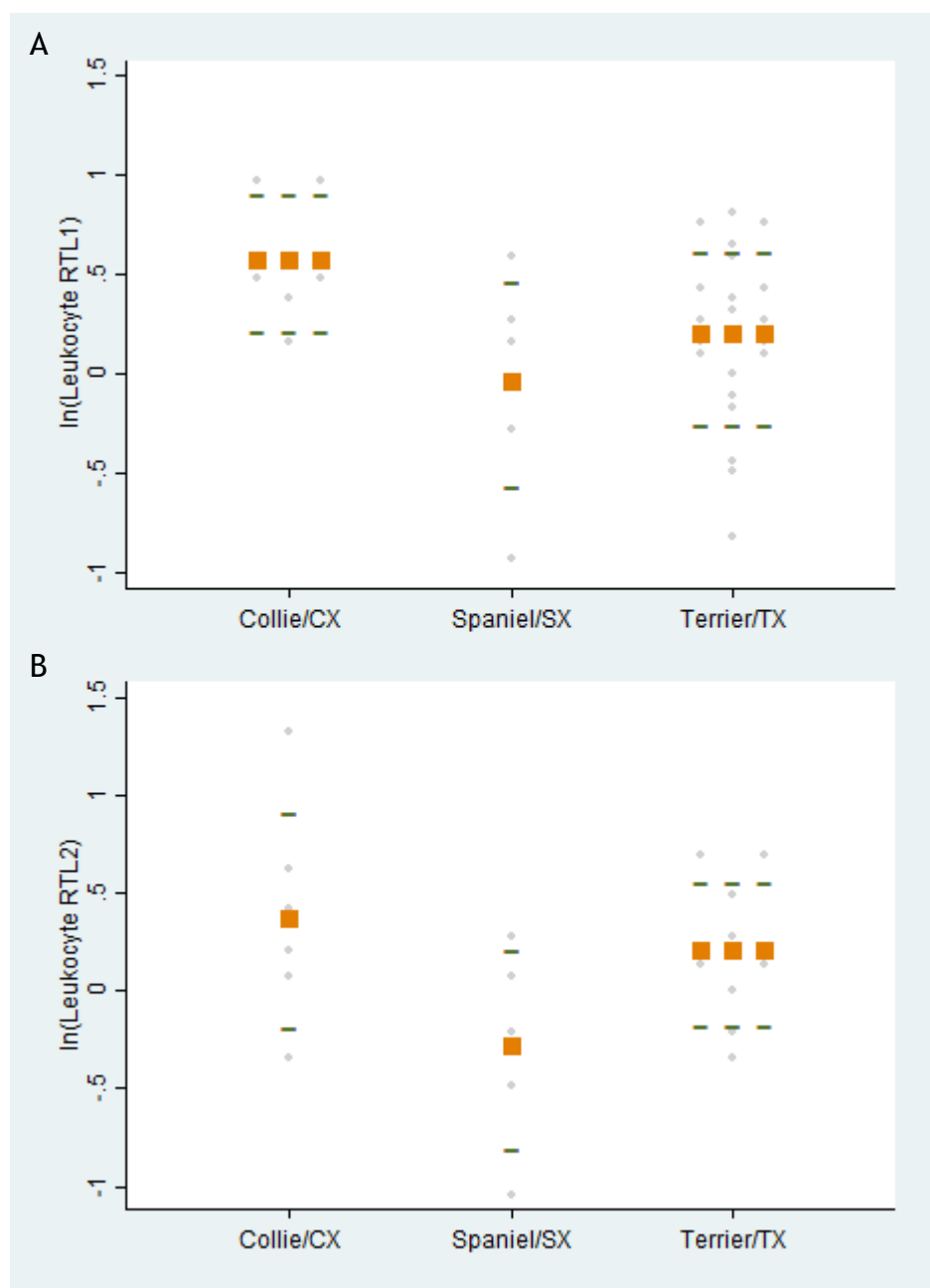


Figure A10.6 Dot plots of natural log of leukocyte RTL1 (A) and leukocyte RTL2 (B) by breed groups

“Collie/CX”= collie and collie cross breeds, “Spaniel/SX”= spaniels and spaniel cross breeds and “Terrier/TX”= terrier and terrier cross breeds. The orange squares represent the mean value and green dashes the 95% confidence intervals. No significant difference was found in leukocyte RTL1 ($p=0.07$) or leukocyte RTL2 ($p=0.08$) between the groups.

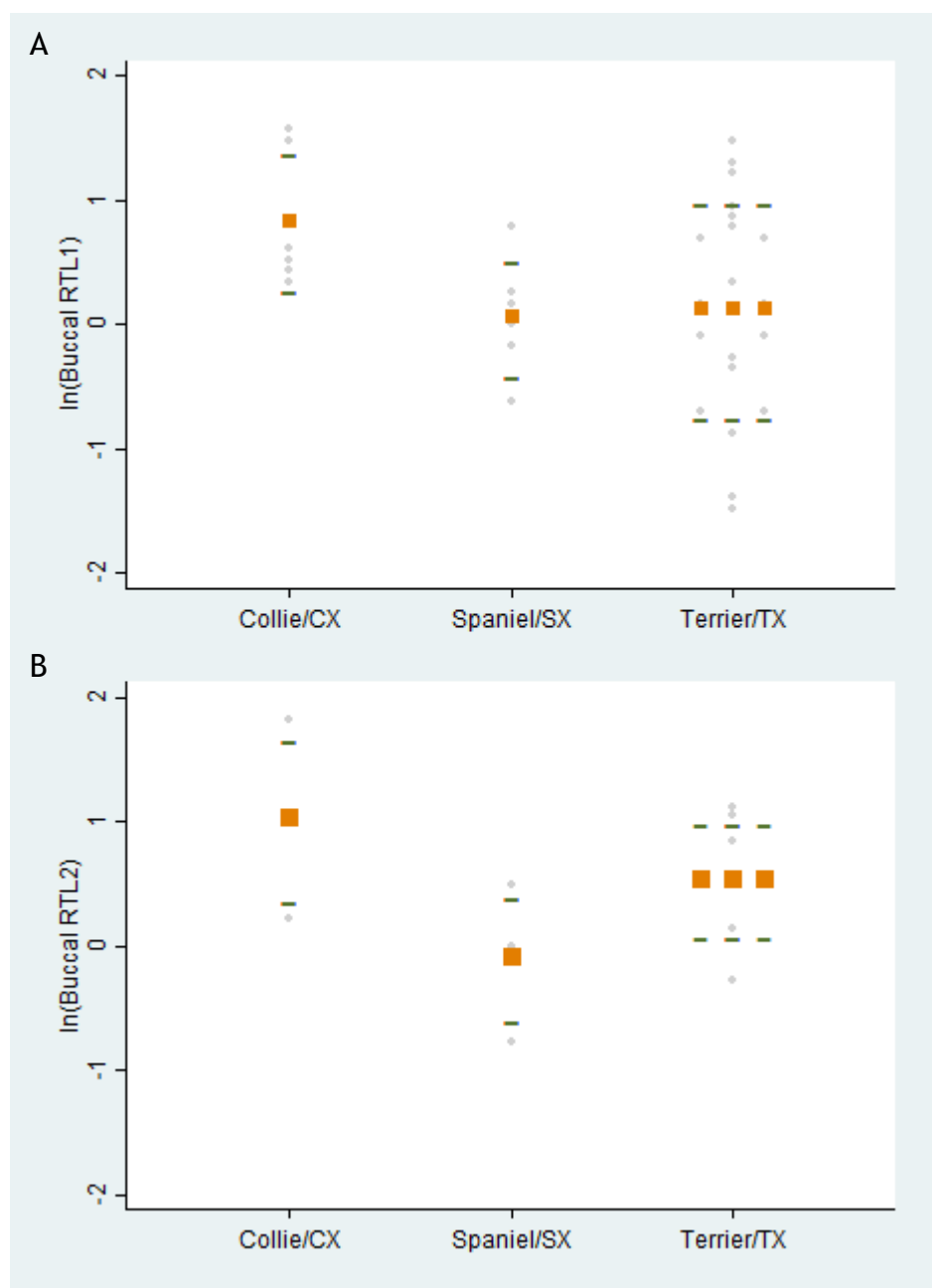


Figure A10.7 Dot plots of natural log of buccal RTL1 (A) and buccal RTL2 (B) by breed groups “Collie/CX”= collie and collie cross breeds, “Spaniel/SX”= spaniels and spaniel cross breeds and “Terrier/TX”= terrier and terrier cross breeds. The orange squares represent the mean value and green dashes the 95% confidence intervals. No significant difference was found in buccal RTL1 ($p=0.13$) between the groups, but a significant difference was observed in buccal RTL2 ($p=0.03$).

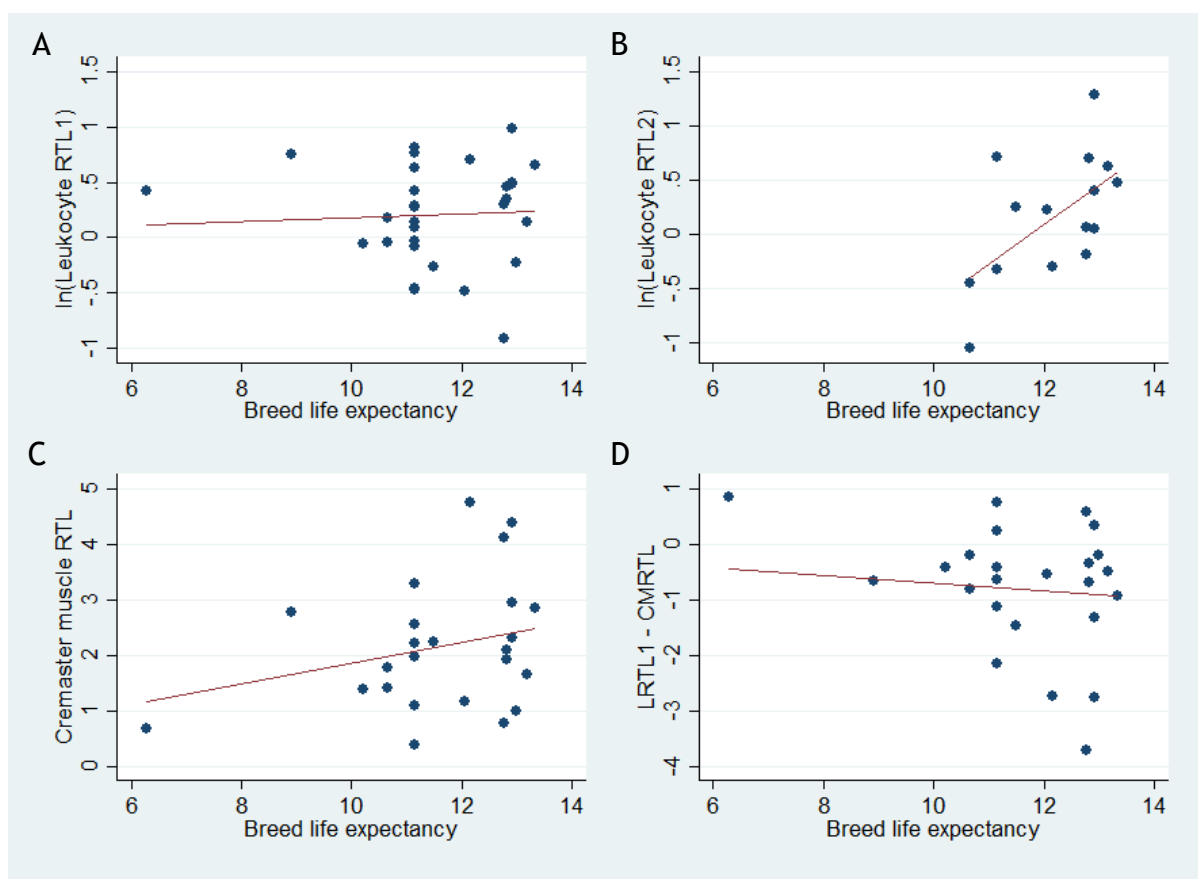


Figure A10.8 Scatterplots with regression line of leukocyte RTL, cremaster muscle RTL and the difference between leukocyte and cremaster muscle RTL by breed life expectancy
 Natural log of leukocyte RTL1 and breed life expectancy (A) ($R^2=0.18$, $p=0.02$); natural log of leukocyte RTL2 and breed life expectancy (B) ($R^2=0.35$, $p=0.02$); cremaster muscle RTL and breed life expectancy (C) ($R^2=0.81$, $p<0.001$); difference between leukocyte RTL1 and cremaster muscle RTL and breed life expectancy (D) (AIC=78.0, $p<0.001$)

Appendix 11: CDKN2A Western blot

Western blot measurement of CDKN2A protein

A subset of the dogs had CDKN2A protein level measured using the same sample of testis from which RNA was extracted, to check concordance between mRNA and protein level measurements. In order to achieve this, a Western blotting protocol needed to be optimised. This technique was selected as it was possible to measure protein in the same tissue sample. Immunohistochemistry could have been performed on the other testis, which had been placed in formalin, but CDKN2A levels could have varied between testes within the same dog.

Table A11-1 Reagents, solutions, consumables & equipment for protein extraction & Western blotting

Anti-CDKN2A antibody	Abcam, Cambridge UK
Anti-histone H3 antibody	Abcam, Cambridge UK
Guanidine hydrochloride	Sigma Aldrich, Gillingham UK
Isopropanol	Sigma Aldrich, Gillingham UK
Non-fat skimmed milk powder	
Nupage LDS Sample Buffer	Invitrogen, Paisley UK
Nupage MOPS SDS Running buffer 20X	Invitrogen, Paisley UK
SeeBlue Plus2 pre-stained standard	Life Technologies, Paisley UK
Tween 20	Sigma Aldrich, Gillingham UK
PBST	0.1% v/v Tween-20 was added to 1X PBS solution
1X MOPS running buffer	To make 900ml running buffer, 45ml of 20X running buffer was added to 855ml dH ₂ O
iBlot Transfer Stack	Invitrogen, Paisley UK
Novex BT Gel 4-12% 1.0MM 12W	Invitrogen, Paisley UK
Gel documentation system	Syngene, Cambridge UK
Gel tank for WB gels	Invitrogen, Paisley UK
iBlot dry blotting system	ThermoFisher, Paisley UK
Sonicating water bath	Guyson International, Skipton UK

Protein extraction

Protein was isolated from tissue using the phenol-chloroform phase obtained in the RNA extraction process. 2 phases remained after removing the upper phase for RNA extraction: the interphase and phenol-chloroform phase. The interphase can be used for DNA extraction and the DNA was pelleted first prior to protein extraction. 300µl of 100% molecular biology grade ethanol was added to each sample and the sample inverted several times to mix, followed by a 3-minute incubation at room temperature. The tubes were centrifuged at 2000 x g for 5 minutes at 4°C to pellet the DNA. The supernatant was removed and placed in a 15ml tube, and protein extracted from the supernatant. 1.5ml isopropanol was added, the tubes mixed by inversion and incubated at room temperature for 10 minutes. The tubes were centrifuged at 10,000 x g for 10 minutes at 4°C to pellet the protein. The supernatant was removed and discarded. The pellet was then washed 3 times, each time by adding 2ml of protein extraction wash solution, incubating for 20 minutes, centrifuging at 7500 x g for 5 minutes at 4°C and then the wash solution removed and discarded. A further wash was performed using ethanol. 2ml of 100% ethanol was added to the protein pellet, the tubes vortexed and then incubated at room temperature for 20 minutes. The tubes were centrifuged at 7500 x g for 5 minutes at 4°C. The pellet was air dried for 10 minutes. 200µl of 1% SDS solution was added to the pellet and pipetted up and down until the protein was resuspended. The tubes were centrifuged at 10,000 x g for 10 minutes at 4°C to sediment any insoluble material and the supernatant transferred to a 1.5ml microcentrifuge tube. The concentration of protein was measured, then stored at -80°C.

Protein quantification

For the protein extraction samples, absorbance was measured at 280nm to calculate the concentration in mg/ml. 1% SDS was used as the blank.

MDCK and HeLa cell lines were used as comparative and control samples. Cells were grown in 10cm dishes (section 2.2). When the cells reached 80% confluency, the dish was removed from the incubator and placed on ice to avoid protein degradation. The media was removed and the cells washed with 8ml PBS. 500µl Nupage LDS sample buffer (Invitrogen, Paisley UK) was added. The cells were scraped off and placed in a 1.5ml screw top microcentrifuge tube. The tube was placed on ice. The cells were then physically disrupted using a 21 Gauge needle and 1ml syringe, by drawing in the fluid and ejecting it back out into the microcentrifuge tube 20 times. The sample was then sonicated in a 4°C sonicating water bath 5 times for 30 seconds each time, with a one-minute gap between each sonication, during which the tube was on ice.

Western blotting

An antibody to canine CDKN2A to purchase could not be found. However, the dog and human protein show similar sequences between 60 and 120 amino acids, so an antibody against this region was selected (Anti-CDKN2A/p16INK^{4a} antibody [2D9A12], abcam Cambridge UK). The expected band size was 40kDa. A loading control antibody with predicted band size of 15kDa was selected, anti-histone H3 antibody (abcam, Cambridge UK), which was listed as reacting to dog samples. Dot blots were performed in order to establish the concentration of the primary antibodies required. Briefly, a dot of testis protein sample with 1:4 NuPAGE LDS sample buffer (Invitrogen, Paisley UK) was spotted onto a square of nitrocellulose membrane and allowed to air dry. One square per primary antibody concentration to be tested was prepared. The membranes were incubated in 10% skimmed milk powder in PBS for 1 hour at room temperature with agitation. Each membrane was then incubated in an antibody dilution at 4°C overnight with agitation. From the information provided with the primary antibodies, anti-CDKN2A antibody was tested at a range of concentrations from 1:500 to 1:2000, and anti-histone H3 was tested at concentrations from 1:1000 to 1:5000. The membranes were washed with PBST and incubated in 1:5000 secondary antibodies (detailed below) and incubated for one hour. The membranes were washed with PBST, PBS and ddH₂O

and scanned using an Odyssey system (LI-COR, Nebraska USA). The primary antibody concentration was decided by determining the lowest concentration at which the protein was detected.

The samples were prepared by diluting to $2\mu\text{g}/\mu\text{l}$ (so $30\mu\text{g}$ would be loaded onto the gel) and adding $5\mu\text{l}$ NuPage LDS sample buffer to $15\mu\text{l}$ of the diluted sample. The samples were then placed in a heat block at 70°C for 10 minutes. The samples to be tested were loaded onto the gels alongside MDCK and HeLa protein samples. The HeLa sample was used as control to calculate the relative expression to, and MDCK was used to calculate the inter-assay variation. Precast 4-12% gradient gels (Novex Bt Gel 1.0MM 12w, Invitrogen Paisley UK) were rinsed twice with ddH₂O and once with 1X MOPS running buffer (Invitrogen, Paisley UK) and set up in a gel tank. The tank was filled with 1X MOPS running buffer. $20\mu\text{l}$ of samples were loaded into wells, alongside $10\mu\text{l}$ SeeBlu Plus 2 pre-stained standard (Life Technologies, Paisley UK). The gel was run at 200V for 50 minutes. The gel was placed in an iBlot system and transfer set up using an iBlot transfer stack (Invitrogen, Paisley UK). Transfer was completed and the resulting membrane placed in PBS for 2 minutes. Blocking was undertaken for 1 hour with agitation at room temperature using 10% skimmed milk powder in PBS. The membrane was then incubated in 1:1000 anti-CDKN2A and 1:5000 anti-HH3 antibodies diluted in 5% skimmed milk powder in PBS overnight with gentle agitation at 4°C . The antibody solution was discarded and the membrane washed with PBST 5 times for 5 minutes each time with agitation. The membrane was then incubated in 1:5000 goat anti-rabbit IgG DyLight 680 and 1:5000 goat anti-mouse DyLight 800 secondary antibodies (Thermo Scientific, Loughborough UK) diluted in 5% skimmed milk powder in PBST for one hour at room temperature. The membranes were protected from light from the secondary antibody incubation step onward, as the secondary antibodies were light sensitive. Following the incubation in secondary antibodies, the solution was discarded and the membrane washed with agitation using the following: PBST 5 times for 5 minutes each time, PBS 3 times for 5 minutes each time and ddH₂O 3 times for 5 minutes each time. The membrane was then scanned using an Odyssey system. The intensities of the bands were obtained

using Image Studio Lite v5.0 software (LI-COR, Nebraska USA). The intensity of CDKN2A was adjusted using the histone H3 loading control and then expression relative to that of HeLa cells calculated.

Unfortunately, the anti-CDKN2A antibody generated multiple bands, and in fact, abcam subsequently were in contact to say the antibody was no longer to be deemed suitable for Western blotting. However, using the band at the expected position, the relative expression was calculated, as the data generated were to support the evidence provided in the main body of work, rather than to stand alone. Using this method, the values generated were plotted against the qRT-PCR generated values in the same dog (Figure A11.1). Correlation testing showed the methods showed good agreement (Pearson's correlation coefficient=0.70, $p=0.001$).

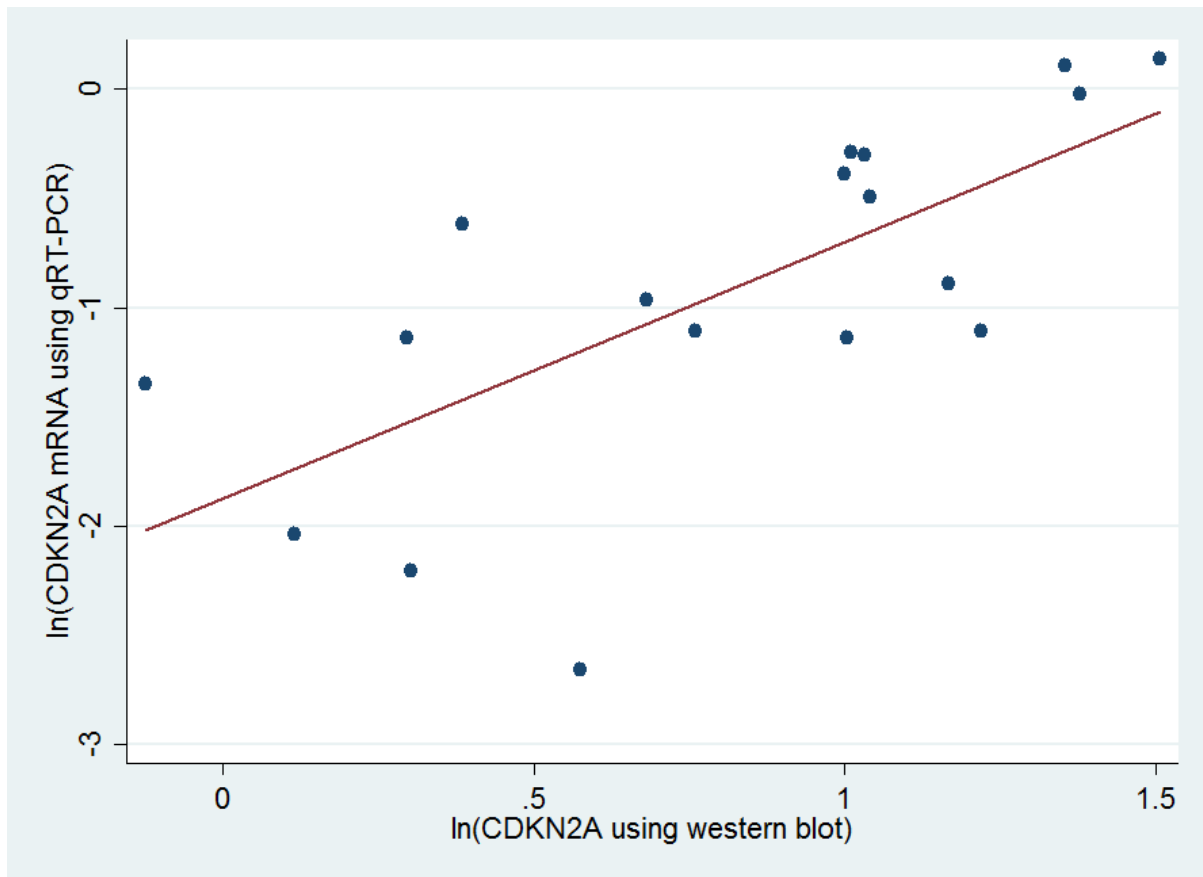


Figure A11.1 Natural log of relative protein expression of CDKN2A and natural log of mRNA expression of CDKN2A in the same testis samples (correlation $p=0.001$)

Glossary- General terms

Biological age	An individual's physiological function relative to the normal physiological progression throughout lifespan
Cross-sectional study	A study of a population using data collected at a single time point
Environmental tobacco smoke	A combination of exhaled smoke from a smoker, sidestream smoke from a lit cigarette and tobacco smoke which lingers in the environment after the cigarette is extinguished
Longitudinal study	A study in which data is gathered from participants on multiple occasions
Pack-years	A numerical value calculated by the number of packs of 20 cigarettes smoked per day by the number of years a person has smoked for

Glossary- Statistical terms

95% confidence interval	An interval constructed from the data, with a 95% chance that the true value lies within that range
Akaike information criterion	A measure of quality of a model relative to other models for a given set of data
Bland Altman plot	A graphical method of comparing two measurement techniques
Coefficient of variation	The ratio of the standard deviation to the mean Shows the extent of variability relative to the mean of the population
F ratio	A ratio of the explained to the unexplained variance in a model
General linear model	A framework for testing how different variables affect different continuous variables
Generalised linear model	As with general linear model, however allows for the response variable having a non-normal distribution
Holm p value correction	Used to reduce the chance of obtaining a statistically significant result when performing multiple tests
Interquartile range	The difference between the upper and lower quartiles of the data Gives a measure of spread

Kruskal Wallis test	A rank-based non-parametric method of testing for differences between two or more groups
Mean	The sum of the data, divided by the number of elements in the set
Median	The value halfway through an ordered data set
Non-parametric	Assumptions are not made regarding the distribution of the data, the data are not normally distributed
One-way analysis of variance	A test to determine if there is a difference in the means of two or more independent groups
p value	The probability of getting a value of the test statistic more extreme than by chance alone
Parametric	Where assumptions are made regarding the parameters of the data Parametric statistics are used for normally distributed data
Pearson's product moment correlation	The strength of linear association between two variables
R^2	The coefficient of determination The proportion of variance in the outcome variable explained by the input variable(s)
Receiver operating characteristic curve	A graph used to determine the diagnostic ability of a test at different cut-off points

Shapiro-Wilk test	A test of normality of data
Spearman's rank correlation	A measure of the strength and direction of association between two ranked variables
Standard deviation	A value describing how much the data vary from the mean
Standard error	A measure of precision of the sample mean
Test sensitivity	The ability of a test to correctly identify those with the exposure/disease/etc
Test specificity	The ability of a test to correctly identify those without the exposure/disease/etc as not having it
t-test	A test of whether the means of two groups significantly differ from one another
Wilcoxon rank-sum test	A test of whether the median of a group is significantly different from another value

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